The guarded brain

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The Guarded Brain

The role of P-glycoprotein at the blood-brain barrier in major psychiatric disorders and antidepressant treatment

Onno de Klerk

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Chapter 1

Introduction

The brain is the most well protected organ in the body. Externally, the brain is protected by the bony skull, whereas it is internally protected from blood borne, potentially toxic substituents by the blood-brain barrier (BBB). The presence of a BBB has been known since the 1880s when Paul Ehrlich demonstrated its presence through the use of a vascular dye. Several decades later, it was Spatz,[1] who proposed a cerebral vascular system resembling the contemporary model of the BBB. It was not until the 1960s that Reese and Karnovsky[2] and Brightman et al.[3] verified this BBB model by localizing tight junctions in the endothelial cells, with the use of electron microscopy.
Later, the uniqueness of the BBB tight junctions and physiology, relating to capillary networks of peripheral organs were addressed by Stuart and Wiley.[4] They showed that embryonic quail brain tissue retained its BBB properties when transplanted into embryonic chick gut tissue. Conversely, capillaries of transplanted quail gut transplanted into embryonic chick brain were leaky to tryptan blue and did not maintain BBB characteristics. These elegant experiments supported the belief that the protective properties of the BBB lie within the endothelial cells and the surrounding tissue. More recent data have emphasized the characteristics of the BBB as a functional barrier.[5] A major aspect of this functional barrier is the way the transport of molecules is performed in tight control by protein pumps located at the luminal side of the capillary wall. Influx is regulated for all molecules, except small, lipophilic and uncharged molecules can freely pass the BBB. The efflux pump P-glycoprotein (P-gp) is however capable of extruding many of these molecules. The physiological role of P-gp presumably involves defence of tissues against xenobiotics or (endogenous) toxic compounds. In the last decades, the role of the BBB and its components have received increased attention in psychopathological conditions.
Chapter 2 gives a general review of the blood-brain barrier, including the main components of the BBB (i.e. endothelial cells, tight junctions, surrounding cells and neurons). Briefly the main function of separate components are explained and a concise description of the three main endogenous transport systems is given. One of these systems is the group of efflux transporters, of which P-glycoprotein (P-gp) is one of the major efflux proteins, which is shortly introduced. In short, we reflect on the altered BBB characteristics in neuropathological conditions.
In *chapter 3* the localization of P-gp at the BBB and consecutively its function, structure and transport activity are described. We focus on the relevance of P-gp in relation to drug disposition of antidepressants and antipsychotics. We discuss the tentative contribution of P-gp modulation (eg. P-gp as susceptibility factor) in affective and psychotic disorders and the possible influence of variarion in the coding gene, *ABCB1* (ATP Binding Cassette family, member B1). Other transporters are also mentioned briefly.
Chapter 4 describes the results of a [^{11}C]-verapamil PET (positron emission tomography) study in (medicated) patients with a major depressive disorder (MDD).

The same protocol was used to assess the [^{11}C]-verapamil in patients with schizophrenia, described in *Chapter 5*. *Chapters 6 and 7 are* a description of two studies using the data of the NESDA cohort (NEtherlands Study on Depression and Anxiety). The effect of variations of the encoding gene for P-gp, *ABCB1*, on different predictors of familial aggregation in depression is discussed in *chapter 6.* This chapter addresses the possible role of *ABCB1* gene variants as susceptibility factor in MDD. In *chapter 7* the association between *ABCB1* gene variants and the incidence and severity of reported side effects on antidepressants is discussed. *Chapter 8* is a description of an animal PET study, which is a continuation of the two human studies (*chapters 4 and 5*) in which we found a regional increase in P-gp activity. The study was set up to differentiate between the effects of antidepressant treatment and disease. The effect of chronic stress on male Wistar rats (as a model for depression) was compared to the effect of chronic administration of venlafaxine, an antidepressant. The effects were measured in vivo using [^{11}C]-verapamil as PET tracer. After the PET the rat brains were collected for western blot analysis and *in vitro* immunohistochemistry. In the following chapter (*chapter 9*) we describe the regional analysis of the effects found in the animal studies. A reanalysis is done for the PET scans, in order to determine whether the effects are global or region specific, the latter possibly indicating a disease specific locus. The results of the *in vitro* studies has not been finished yet, but may denote regional or global changes in protein expression. The future perspectives, relating to further research on the various roles of P-gp may play in psychiatry, are outlined, including its role in drug disposition and a protective role as part of the BBB.

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Chapter 2

The blood-brain barrier

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Introduction

The central nervous system requires a perfectly regulated environment, for optimal activity. The main contribution to this homeostasis is accomplished by the BBB, by protecting and isolating the nervous tissue from too strong fluctuations in nutrients, hormones, metabolites, and other solutes. The microenvironment within the brain is precisely controlled by the BBB, and neuronal transmission is secured in this way.[1] It also protects against the direct influence of potentially harmful endogenous or exogenous substances. The BBB has to be a stable structure to exert its protective function, but on the other hand the BBB requires the ability for plastic changes,[2] in order to adapt to fast changing conditions. Several areas, such as the pineal body and the pituitary, both small endocrine glands in the brain, are not ”behind” the BBB, but secrete their hormones directly into the systemic circulation.

The protective function of the BBB is exerted in two ways. Until recently the BBB was largely viewed as an anatomical barrier, preventing the passive diffusion of solutes from blood to the brain. In addition to being a physical barrier, the BBB is a complex transport and metabolic barrier due to its highly reactive and dynamic endothelium. The major components of the anatomical barrier are the specialized non-fenestrated tightly-joined endothelial cells with tight junctions (see figure 2.1).[1,3,4] In the following paragraphs we will discuss these main components of the BBB.

Components of the BBB

Endothelial cells

Brain endothelial cells (EC) are unlike other endothelial cells in the body. Compared to the leaky capillaries formed by mesenteric endothelium, the brain endothelium is 50-100 times tighter, as indicated by the transendothelial electric resistance (TEER).[5] They differ phenotypically by a lack of fenestrations and the presence of tight junctions. The rate of pinocytosis is minimal and free membrane diffusion applies mainly to small lipophilic molecules like ethanol or nicotine.[6] The ECs are surrounded by a basal lamina, which further restricts the microvascular integrity.[7] ECs are rich in mitochondria, necessary for their high metabolic demands. Specific enzymes expressed by ECs (monoamine oxidases, epoxy hydrolase, endopeptidases, etc.) are important elements, constituting the so-called metabolic barrier, and participate in the regulation of brain penetration of drugs and their metabolism.[8-11] ECs have an extensive transport system on their surface, which is carrier mediated or receptor mediated.

Tight junctions

Tight junctions are the closely associated areas of two cells whose membranes join together forming a virtually impermeable barrier to fluid. This is accomplished by their structure, as it is composed of a branching network of sealing strands, a complex of transmembrane (junctional adhesion molecule-1, occludin, and claudins) and cytoplasmic proteins.[1,12] Each strand acts independently from the others. Tight junctions prevent the passage of molecules and ions through the space between cells, the so called paracellular flux. In order to pass the blood-brain barrier molecules must enter the endothelial cells. Together with the endothelial cells, the tight junctions play the most substantial role in maintaining the BBB.[13]

Astrocytes, microglia and pericytes

Astrocytes, microglia and pericytes surround the endothelial cells, and form the "second line of defence" in the BBB. Astrocytes are star shaped glia cells, that perform many functions, including the biochemical support of endothelial cells. Their end-feet, encircling the endothelial cells aid in the maintenance of the blood-brain barrier, as they regulate the homeostasis of brain water and electrolytes.[1,14] In addition, they interconnect endothelial cells with surrounding neurons. Astrocytes secrete proteins that can have an opposite effect on neurons and endothelial cells. For example, thrombospondin, an astrocyte-derived protein stimulates neurogenesis on the one hand, while it counteracts the effects of angiogenesis.[15] Current in vitro cellular models for the study of BBB function often incorporate astrocytes with endothelial cells. Studies have shown that when astrocytes are removed from an in vitro BBB model, an increased permeability is observed.[16] Many transporters (see below) are under the control of astrocytes.[17] In pathological conditions, astrocytes mediate the immune response, they synthesize proinflammatory cytokines and chemotactic factors, that eventually trigger the breakdown of the BBB.[12] The pericyte, another foremost cell type in the CNS, intimately embraces the brain endothelial capillary. Less is known about their role at the BBB, although they seem to stabilize the formation of capillary-like structures when they are added to a co-culture of astrocytes and endothelial cells.[18] Microglia are omnipresent throughout the brain parenchyma. They are the immune effectors in the CNS, as they can release a large number of immunoregulatory, inflammatory, and cytotoxic mediators.[19] They surround the brain capillaries and stay in a resting state until they are activated, which can be induced by a variety of stimuli.[20,21] Figure 1 shows the main components of the BBB.

neurons

Neurons lie in close proximity of the brain capillaries and their needs, including protection and the unrestricted provision of nutrients, are optimally met. Neuronal tissue needs an abundant supply of oxygen. At rest, 80–92% of its ATP comes from oxidative metabolism of glucose.[22] Given the dynamic nature of neural activity and the considerable metabolic needs of nervous tissue, the microcirculation of the brain must be highly responsive to the tissue it supplies. Iadecola found that blood flow was increased in response to local neuronal activation and proposed a role for nitric oxide as a messenger between blood and neuronal tissue.[23] This "metabolic coupling" of regional brain activity to blood flow is the basis of functional neuroimaging.[24] However, data from positron emission tomography (PET) and functional MRI studies indicate that during short-term functional activation, cerebral blood flow and cerebral metabolism (oxygen consumption) are not directly linked.[22,25]

Transport across the BBB

There are two tightly controlled ways of transport for molecules and cells across the BBB (see figure 2.2). The paracellular route, or junctional route, is restricted by the interendothelial tight junctions. Tight junctions not only restrict paracellular flux, but also maintain polarity of enzymes and receptors on luminal (blood) and abluminal (brain) membrane domains.[26,27] Due to the presence of tight junctions, only lipid-soluble substances or those transported through an active mechanism can cross the BBB.[3,28]

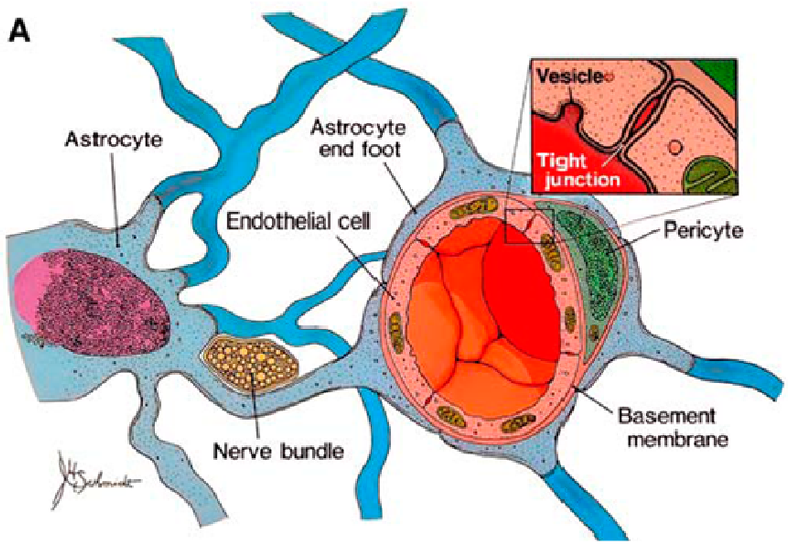
The transendothelial route further restricts the passage of molecules by three distinct transport systems. Small lipid-soluble molecules can penetrate the brain EC through diffusion, unless their molecular weight exceeds 400-600 Da.[29] All other transport to the brain is via endogenous catalyzed transport systems on the capillary membrane. As we will describe below, three main transport systems function at the BBB: (1) carrier mediated transport, which relies on a molecular carrier present on both the luminal side and the abluminal side of the BBB. (2) receptor-mediated transport for endogenous large-molecule peptides such as insulin[30] or transferrin[31] and (3) active efflux transporters such as P-glycoprotein and many other active efflux transport systems within the BBB (see figure 2.2).

Carrier mediated transport

Carrier mediated transport is a highly selective form of transport for small molecules such as ions, glucose and amino acids. Examples of carrier mediated transport systems include the GLUT1 glucose transporter, the OAT (organic anion transporter) and organic anion transporting polypeptide family (OATP), the monocarboxylate transport

family and the LAT1 (large neutral amino-acid transporter) [32]. This form of BBB transport is a saturable process which can be unidirectional or bidirectional. Carrier mediated channels can be gated and can function ion and energy independent, like the LAT1.[33] The membrane spanning pores are highly stereospecific. The substrate forms a complex with the carrier in order to be translocated to the opposite side of the membrane.[34] L-DOPA is such a drug, that utilizes the LAT1 to enter the brain. Once transported through the BBB, L-DOPA is reformulated to dopamine.[35] Another example is valproic acid, that is delivered to the brain via a medium-fatty chain transporter.[36] Uptake of valproic acid was reduced in the presence of medium-chain fatty acids, but not short-chain fatty acids, indicating that valproic acid is taken up by a transport system for medium-chain fatty acids. The monocarboxylate transport family also appears to be involved in the transport of valproic acid, possibly as efflux transporter.[32]

Figure 2.1



cross section of a brain capillary, depicting the main components -

Receptor mediated transport

Another main transport mechanism at the BBB is receptor-mediated transport, which involves a vesicular trafficking system of the endothelial cells (fig. 2). The influx of several brain nutrients like leptin[37] and insulin[38] occurs by this form of transport. Circulating molecules are bound to a specific receptor at the plasma membrane, to form a receptor-ligand complex. When the ligand is bound to the receptor, the process of invagination is initiated. Dependent on subsequent intracellular processes, the vesicles are either sent to the basolateral side of the cell, where they are released, or the

complex is dissociated from the ligand in the cell. A similar transport system is called 'absorptive-mediated transcytosis' which does not require specific binding to a receptor. Instead, binding is non-specific, for example to negative charges on the plasma membrane. The receptor mediated transcytosis offers a promise as drug vector for drug delivery into the brain.[39]

Efflux transport

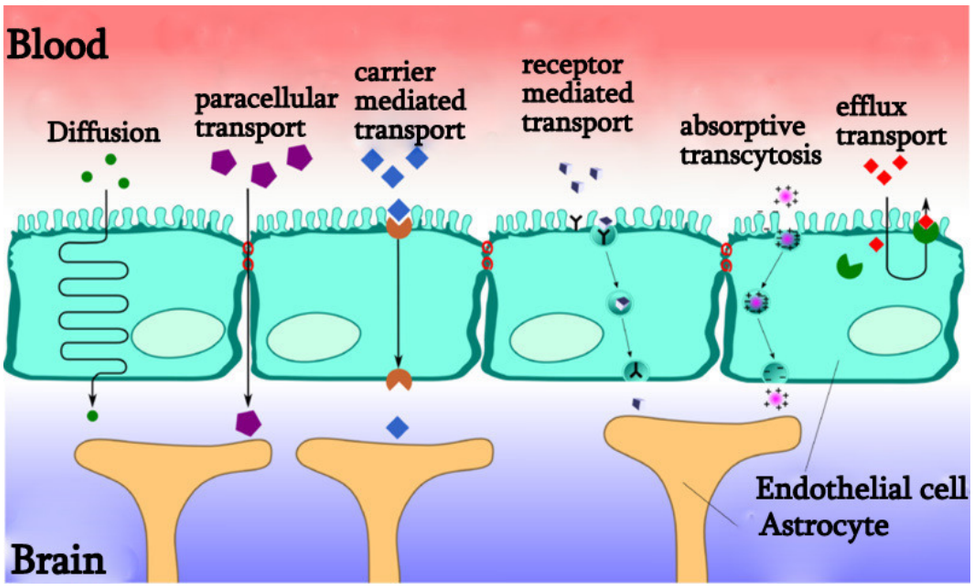
Uncharged, small lipophilic compounds enter the brain much better than other molecules, because they can diffuse passively across the endothelial cells. However, an ATP-driven efflux system exists at the luminal wall of the capillary wall, that can extrude a variety of structurally diverse drugs, drug conjugates and metabolites, and other compounds from the cell. These efflux proteins involved belong to the superfamily of ABC (ATP Binding Cassette) transporters, an extensive and functionally highly diverse family of membrane transporters. Export of these compounds occurs in an active, ATP-dependent manner, and can take place against a considerable concentration gradient. [40,41] Thus far, 49 human ABC transporter genes have been identified.[42] They are classified into eight subfamilies (ABCA-ABCH) [43], P-glycoprotein (P-gp, ABCB1), multidrug resistance protein ABCC subfamily, (formerly denoted as MRP) ABCC4 and ABCC5 (and possibly ABCC1 and -2, see below)[42,44-46] and the breast cancer related protein (BCRP, ABCG2) have been localized in the apical membrane of the brain endothelial cell and have a role in efflux at the BBB. Of these, P-gp is the best studied transport protein. Its most striking property is transport of a wide range of structurally different substrates, including many CNS drugs. As a consequence, the net penetration of substrate drugs and other substrate compounds from the blood into the brain tissue can be dramatically decreased. Various members of the ABCC family show considerable differences in their tissue distribution, substrate specificity, and proposed physiological function. These proteins play a role in drug disposition and excretion and thus are implicated in drug toxicity and drug interactions. ABCC primarily transports anionic compounds, such as glutathione S- conjugates and oxidized glutathione.[47] Furthermore, ABCC transport appears to be dependent upon intracellular glutathione.[48]

Alteration of BBB permeability in neurodegenerative disorders

Altered BBB integrity is an early event in many neuroinflammatory conditions, including meningitis, multiple sclerosis, epilepsy and neuropsychiatric disorders.[2,49-54] In contrast, Bartels et al. suggested BBB breakdown to be a late event in Parkinson's and related diseases.[55] Alteration in BBB permeability occurs secondary to cell damage in trauma, ischemia and stress. An inflammatory response results, with a release of proinflammatory cytokines such as TNF-α (tumor necrosis factor), IL-1β (interleukine-1β), IL-6.[51,56,57] These three appear to be the main cytokines involved in the regulation of the acute phase response, by up-regulating cell adhesion molecules, activating T-

cells and B-cells and stimulating the release of acute phase proteins. These cytokines and toxic factors such as nitric oxide promote neurodegeneration and may also activate astrocytes which will further affect the integrity of the BBB. Features of BBB disruption include increased vesicular transport across cells, separation of tight junctions, swelling of astrocytes, activation of microglia cells and disintegration of the capillary basement membrane. Cytokines represent an important communication pathway for the brain and immune system, they are also intimately involved in the stress response. For example, proinflammatory cytokine production in the brain is upregulated following exposure to physiological or psychological stressors.[58] In response to stress, proinflammatory cytokines have been shown to modulate transport systems at the BBB, e.g. the voltage gated K+ channels,[59] lactoferrin transport[60] and the efflux pump P-gp.[61-63]

Figure 2.2



Transport mechanisms at the blood-brain barrier

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Chapter 3

The role of P-glycoprotein in psychiatric disorders: a trustful guard of the brain?

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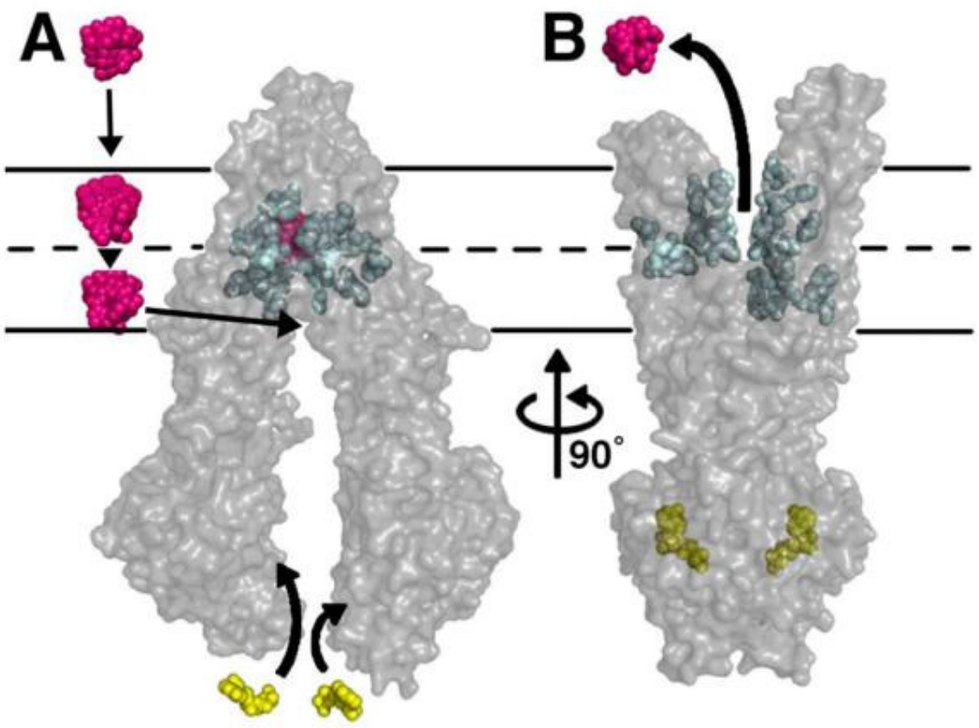
Abstract

A major component in the protection of the brain against blood-borne toxic influences is the multispecific efflux pump P-glycoprotein. This pump, a 170 kD protein, located at the luminal side of the capillary endothelial cells, has a large capacity and is capable of extruding a wide array of structurally divergent substrates. The brain uptake of the majority of antidepressants and antipsychotics, as well as many other psychotropic drugs and endogenous compounds is hampered by the activity of P-glycoprotein. In this review we discuss the current state of knowledge concerning the role of P-glycoprotein on pharmacokinetics of psychiatric drugs and the impact of modulation of P-glycoprotein on major psychiatric disorders. Relevant issues in reference to the function of P-glycoprotein and other efflux pumps in the blood-brain barrier related to mood disorders and schizophrenia are addressed, such as a possible role of P-glycoprotein as a susceptibility factor in depressive disorders and psychotic disorders.

Introduction

The central nervous system (CNS) requires a perfectly regulated environment, for optimal activity. The main contribution to this homeostasis is accomplished by the blood-brain barrier (BBB), by protecting and isolating the nervous tissue from too strong fluctuations in nutrients, hormones, metabolites, and other solutes. Due to the presence of tight junctions, preventing the passage of molecules and ions through the space between cells, all substances must pass through the endothelial cell before entering the brain.[1,2]

Figure 3.1



Model of substrate transport by P-gp. (A) Substrate (magenta) partitions into the bilayer from outside of the cell to the inner leaflet and enters the internal drug-binding pocket through an open portal. The residues in the drug binding pocket (cyan spheres) interact with a substrate in the inward facing conformation. (B) ATP (yellow) binds to the nucleotide binding domains causing a large conformational change presenting the substrate and drug-binding site(s) to the extracellular space. In this model of P-gp, exit of the substrate to the inner leaflet is sterically occluded providing unidirectional transport to the outside. Reprinted from Aller et al. with permission[12]

This transendothelial route further restricts the passage of molecules by distinct and highly selective transport systems, present on the luminal side of the endothelial cell. Only small lipid-soluble molecules can penetrate the endothelial cell through diffusion, unless their molecular weight exceeds 400-600 Da.[2]
During the last decade it has become clear that multispecific, xenobiotic transporters play an important role at the blood-brain barrier (BBB). Of all efflux pumps discovered at the BBB so far, P-gp is the best described. It has been shown that P-gp exerts an important influence on the penetration of psychoactive drugs at the BBB.[3,4] P-gp is found at the luminal side of the endothelial cells, where it extrudes hydrophobic compounds from the cell. It was discovered in 1976 in drug-resistant ovary cells from Chinese hamsters.[5] Human P-gp is encoded by the multidrug resistance gene (MDR1), which is now denoted as ABCB1 (ATP Binding Cassette gene B1). P-gp contains 1280 amino acids and has a molecular weight of 174 kD.[6]

Localization and function of P-gp

Cerebral P-gp is not only present at the BBB, but also at the blood-CSF barrier.[7] Apart from the CNS, P-gp is present in kidneys, gut and other organs that have an epithelial lining. Interestingly, while in normal conditions P-gp is not found in neurons, neuronal P-gp expression was reported in pathological conditions, such as refactory epilepsy, cortical dysplasia and glioneuronal tumors.[8-10] The physiological function of ABCB1 has not been unambiguously identified yet,[6,11] but it involves the protection of the brain from compounds that have gained access to the circulation. The fact that it is being expressed in damaged neuronal tissue as well as in malignancies, affirm such a function.[8,9] It needs to be considered that xenobiotic or drug concentrations in the brain might also be affected by intestinal P-gp limiting oral bioavailability.

Its imposing ability to transport hundreds of structurally divergent drugs, natural products and peptides, render this protein a fascinating molecule. P-gp is composed of two transmembrane bound domains, each consisting of 6 transmembrane helices and two nucleotide binding sites, that hydrolyze ATP, enabling substrate transport (see figure 1).[12] P-gp substrates are known to partition into the lipid bilayers and accumulate to high concentrations. In the inner leaflet of the lipid bilayer the compounds can then get in contact with the drug binding pocket of the transporter.[11,12] Initiated by ATP binding a conformational change of the transporter molecule then brings the drug binding site to the outer leaflet or extracellular surface, thereby promoting unidirectional transport to the extracellular space.

In vivo imaging of P-gp at the BBB

In the last decade considerable effort has been put into gaining further insight into the role of P-gp under pathophysiological conditions. Different substrates of P-gp have

been radiolabeled and imaged using single-photon emission computed tomography (SPECT) and positron emission tomography (PET).[13]

A P-gp tracer that meets the criteria of a useful ligand is a selective substrate for P-gp, produces a good signal after P-gp blockade and generates few radiolabeled metabolites that contribute to the PET signal. The principle of PET imaging of P-gp activity amounts to measure the difference in cerebral uptake of a radiolabeled P-gp substrate (i.e. radioligand) under two different conditions, for example major depressive disorder vs. healthy control, or before and after drug inhibition. The function of P-gp can be quantified by calculating the distribution volume (V_T) of the ligand, which inversely reflects P-gp function.[14] V_T is an estimate of the brain tissue-blood partition coefficient of activity of the radiotracer at equilibrium and is defined as the influx rate constant K_1 over the efflux rate constant k_2. Several radiolabeled P-gp substrates are available for PET, such as [^{11}C]-verapamil and [^{11}C]-carvedilol, [^{11}C]-loperamide and [^{11}C]-desmethyl-loperamide.[15-17] Of these, the in vivo studies with [^{11}C]-verapamil outnumber all other ligands and it is the only tracer used in clinical studies. The (R)-enantiomer of [^{11}C]-verapamil can be considered superior to the racemic mixture.[14,15,19] Although the feasibility of in vivo measurement of P-gp function by [^{11}C]-verapamil-PET (VPM-PET) has been confirmed by several research groups,[14,18,19] it is not an ideal tracer, because its brain uptake is low. The production of several metabolites of the parent compound [^{11}C]-verapamil does not appear to be a problem, since the main metabolite is also a P-gp substrate.[14] Several strategies have been launched in search of an optimal tracer, that is capable of detecting subtle changes in function (in particular increased P-gp activity). The development of a radiotracer that is not a substrate but works as a pure inhibitor, could bypass this problem, but so far the results of this strategy are ambiguous, possibly due to the fact that these inhibitors have affinity as a substrate too.[20] Several new imaging strategies may still hold the unfulfilled promise of a method to study drug interactions with P-gp. For example, radiolabeling of a new drug candidate after administration of an inhibitor, or a double PET scan to evaluate the effect of a P-gp modulator. Another strategy that could work is the co administration of a P-gp inhibitor, that can increase the baseline signal.[21]

Relevance of P-gp in relation to antidepressants, mood stabilizers and antipsychotics

Different in vitro approaches and in vivo models (ABCB1ab (-/-) knock-out mice) have been used to assess the impact of P-gp on pharmacokinetics of psychiatric drugs. In vitro data have indicated that most antidepressants and antipsychotics have affinity for P-g (table 3.1).[22-26] Many of the *in vitro* data are contrary, which is inherent to the different methods and materials used.

Most research focusing on the inhibitory effects of antidepressants and antipsychotics has been done with various cell lines expressing (recombinant) human P-gp. As a measure of the P-gp-inhibitory potency of the drug a prototypic P-gp substrate, such as calcein-AM or Rhodamine123, was often used. The concentration needed to displace 50% of the prototypic compound (called IC_{50} or EC_{50}) was used as measure of the inhibitory effect on P-gp activity.

For most compounds, the ability to inhibit P-gp has been compared to a typical comparator, such as verapamil or cyclosporine A. (tables 3.1-3.3). It must be considered that these inhibitors significantly differ in their potency to affect P-gp transport function.[27] For example, haloperidol appeared to be a weak inhibitor compared to PSC833,[28] whereas it proved to be a much stronger inhibitor in comparison to verapamil and ivermectin.[29]

Some antidepressants have only shown inhibition without demonstrated substrate affinity (i.e. desipramine, imipramine, reboxetine).[24,25] Besides, the translation of *in vitro* results to the human case could be complicated by differences in substrate specificity for P-gp across species.[30] A more reliable method of studying affinity for P-gp is based on studies in knock-out mice, which lack the ABCB1ab genes encoding P-gp. Affinity for P-gp has been demonstrated in knock-out mice for most of the common antidepressants[23,31-35] and antipsychotics (tables 3.1-3.2).[31,36-41]
Despite the differences in the experimental set-up that partly explain the controversies regarding the interaction between P-gp and antidepressants and antipsychotics, it is feasible to say that the majority of the antidepressants and antipsychotics have shown (mostly weak) affinity as a P-gp substrate and that most have a weak inhibitory effect on P-gp *in vitro*. Some authors have argued that the inhibitory effect of antidepressants on P-gp is clinically irrelevant,[25,26,34] since the drug concentrations used in most studies, necessary for P-gp inhibition were far above the therapeutic dose range. On the other hand, others have suggested that some antidepressants may be strong inhibitors in vivo.[23,42,43] For antipsychotics comparable differences between the individual drugs have been described.

The impact of cerebral P-gp in man for the bioavailability of psychoactive drugs cannot be satisfactorily deducted from the *in vitro* data. It needs to be considered that antidepressants and antipsychotics can generally access the brain and therefore can not be high affinity substrates of P-gp like compounds that do not exhibit any relevant brain penetration based on its interaction with P-gp. Moreover, CNS active drugs are in general characterized by a fairly high lipophilicity. Recent data demonstrated that it can be more difficult to detect an interaction with P-pg, when drugs pass membranes efficaciously by rapid diffusion due to their lipophilic characteristics.[44]

Most of the antidepressants and antipsychotics, as well as several mood stabilizers demonstrated a significantly greater, albeit small brain/plasma ratio in ABCB1a knockout mice compared to wild type mice (mean = 1.8 for antidepressants; 4.9 for antipsychotics) (see table 3.1-3.3). The value of the studies using the knock-out mouse model is limited by the fact that a one dose model is mostly used, representing only the acute modulatory effect on P-gp. There are only two reports on long term treatment on P-gp activity *in vivo*.[45,46] For amitriptyline, it was demonstrated that the brain uptake in WT and KO mice was equal for the parent compound (amitriptyline), whereas a much higher cerebral concentration was measured for its metabolites in the knock-out mice. It was suggested that chronic administration of amitriptyline induces up-regulation of the P-gp pump, thus inhibiting its own access to the brain. In an elaborative study they refined their results and demonstrated that 4 hours after a single dosis of amitriptyline the initial differences (present at one hour post injection) in cerebral uptake had disappeared, in contrast to the metabolites, pleading against an up-regulatory mechanism.[47] Differences in cerebral uptake between the parent compound (amitriptyline) and its metabolites can also be explained by a differential affinity of P-gp for the respective molecules. Miura et al. demonstrated that small molecular changes can turn a strong P-gp substrate into a weak one.[48] Another notion is that the effect of a non-substrate on P-gp activity can apparently vary over time.[23]

In spite of the findings of the preclinical work on the role of P-gp in the uptake of drugs used for the major psychiatric disorders, there are arguments pleading against a significant role for P-gp. For example, antipsychotic drugs like risperidone and haloperidol may significantly differ in their interaction with P-gp, but have a comparable clinical efficacy. Because direct evidence for a major role of P-gp in pharmacokinetics has been lacking, CNS side effects of drugs with potential P-gp inhibiting effects may erroneously have been attributed to other causes. Cytochrome P450 3A4, a major drug metabolizing enzyme, shows a striking overlap in substrate specificity with P-gp.[49,50] Several CNS effects have been ascribed to interactions between drugs concurrently inhibiting Cyp3A4, without notice of a potential concomitant P-gp inhibiting effect of these drugs in vitro.[51-53]
Cortisol is a substrate of P-gp as well. Its uptake into the brain is thwarted by P-gp.[54,55] It has been well established that depression coincides with dysregulation of the HPA axis, characterized by negative feedback inhibition and elevated cortisol levels.[56] As some of the in vitro data have suggested that P-gp is inhibited by the action of antidepressants,[57] it has been hypothesized that antidepressants exert their antidepressant effect partly by the inhibition of P-gp, leading to an intracerebral cortisol increase and normalization of the HPA axis. However, antidepressants appear to have an insignificant effect on plasma cortisol levels.[58] Besides, cortisol entry to the

brain is not regulated by P-gp only, as Mason et al. found in mice.[59] Concluding, data supporting the hypothesis that P-gp inhibition plays a role in normalisation of a hyperactive HPA-axis, are lacking.

Mood stabilizers belong to different drug classes. Lithium is the drug of first choice in the treatment of bipolar disorders. Although lithium itself is not a substrate of P-gp, lithium shares many properties with magnesium (Mg^{2+}), such as a synergistic effect on the Na/K ATP-ase.[60] Mg2+ appears to play a key role in P-gp mediated efflux through ATP hydrolysis.[61] It may thus be conceived that lithium has a similar impact on the function of P-gp. Other drugs registered as mood stabilizers include the anti-epileptic drugs carbamazapine, valproic acid and lamotrigine. These agents appear to have no or at most a weak affinity for P-gp (see table 3.3). Reports on the effect of different antiepileptic drugs on P-gp expression in brain capillary cell lines have been equivocal.[62-64]

Modulation of P-gp at the BBB by stress (in vitro studies)

An understanding of the physiological regulation of P-gp is key to therapeutic strategies in the treatment of psychiatric disorders, since most of the drugs used for depression and psychosis seem to be P-gp substrates. Stress is the hallmark of many psychiatric diseases, including depression and psychosis, and it appears that all sorts of stress responses evoked by a variety of environmental stimuli, such as cytotoxic agents, heat shock, irradiation, genotoxic stress and inflammation are able to influence either the expression or the activity of P-gp.[65] P-gp is regulated at various levels of expression including DNA, mRNA and protein.

There is increasing evidence for the role of cytokines in the pathogenesis of depression.[66] Proinflammatory cytokines are produced by different immune cells upon presentation of an antigen and their secretion is believed to be the prime event in the subsequent neurophysiological responses taking place during immune stimulation. Several animal models are applied,[65,67] and although results of the in vivo studies are somewhat conflicting, most demonstrated that P-gp expression and activity can be involved in different ways during an inflammatory episode, the degree and direction of the change in P-gp activity depending on the model and the inflammatory mediator used.[67,68]

Several in vitro studies have tried to extricate the signalling pathways of the involved cytokines and other chemotactic compounds leading to a functional change of P-gp at the BBB. Hartz and colleagues define a pathway through which P-gp is acutely modulated. They describe a sequence of events, starting with TNFα (tumor necrosis factor), releasing endotheline-1, that in turn activates nitric oxide synthase and then

protein C kinase, ultimately reducing P-gp transport.[69,70] The same group showed that this regulation is biphasic: after an initial rapid decrease of P-gp activity following exposure to TNF and endotheline-1, an increase in transport activity was found at 6 hours post exposure. Similar data are presented by others. For example, the results by Tan and colleagues showing an increase in P-gp expression following BBB breakdown by activated T-cells, suggest both a role in immune cell mediated cytotoxicity and a counterregulatory role in promoting cell survival and maintaining BBB integrity. They hypothesize that the latter role may be relevant in later stages of the inflammation.[71] *In vivo* studies focusing on such a biphasic response in cytokine regulation and in P-gp activity in depressive or psychotic disorders are warranted and can be best worked out in animal models.

Depressive disorder, schizophrenia and stress-related disorders and P-gp

A few studies have focused on BBB characteristics in relation to psychological stress. Rats subjected to perinatal stress had an increased permeability as measured by the uptake of Evans blue.[72] Acute stress (e.g. immobilization stress or forced swim) increases BBB permeability likewise.[73-75] In respect to the effect of chronic stress on BBB function, studies are lacking, but in major depressive disorder (MDD) BBB dysfunction has been suggested as potential mechanism.[76]

If P-gp is a susceptibility factor in stress related disorders, animal models could be used to demonstrate their modulatory role in stress. To date, evidence is sparse. In one study, describing the anxiolytic-like effect of tariquidar (a P-gp inhibitor) in mice sensitized by a mild stressor, it was suggested that P-gp inhibition led to an increase of corticosteroids, which, in turn would enhance the negative feedback control of the HPA axis.[77]

In two PET studies with [^{11}C]-verapamil, an increased function of P-gp at the BBB in temporal and frontal areas was found both in a group of medicated patients with MDD and schizophrenia.[78,79] Caution in the interpretation of the results of both studies is justified, since the findings were complicated by the use of either antidepressants or antipsychotics. The increase in P-gp function might be a result of the disorder itself or a result of antidepressant treatment. A sequel to these studies was set up to disentangle the possible contribution of antidepressant therapy and MDD. Rats were either subjected to chronic stress or to a continuously administered antidepressant. Using [^{11}C]-verapamil PET as a measure of P-gp activity, an indication was found for decreased P-gp activity in the stressed rats, whereas venlafaxine appeared to have an opposite effect on P-gp activity.[80] A confirmation study correlating the in vivo results from both studies to P-gp expression at protein and mRNA level is under way. Although the in vivo imaging study may provide an indication for a susceptibility role

of P-glycoprotein in depressive disorders, in the light of the limited evidence so far, it is impossible to interpret the findings without great restrictions.

The role of ageing and neurodegeneration in P-gp functionality

Although the status of both MDD and schizophrenia as neurodegenerative disorders is tenuous, both disorders have neurodegenerative features. In depression, it has been hypothesized that neuroinflammation ultimately leads to neurodegeneration.[81] The coincidence of structural brain changes and decreased regional blood flow has been well established in MDD as well as in schizophrenia. Loss of the integrity of the blood-brain barrier is a cardinal phenomenon in many neurodegenerative disorders including Alzheimer dementia. In the PET studies on Parkinson's disease a role for P-gp in neurodegeneration has been suggested, but this is based on small differences in tracer uptake between patients and control group.[82]

The decline in function of P-gp in old age, which has been indicated by [^{11}C]-verapamil-PET studies will certainly accentuate the problems encountered in pharmacotherapy in the elderly.[83,84] The decreased P-gp activity might be linked to the pathology in late-life depression, such as the cognitive changes, but a major role in the etiology of MDD at old age is refuted by the fact that the incidence of MDD declines with age.[85]

Polymorphisms of P-gp and their effect on function.

Since the first report on a polymorphism in the ABCB1 gene, the quest for more variations in the DNA sequence explaining phenotypical differences has not stopped. Substantial progress has been made in identifying single nucleotide polymorphisms (SNPs) in the entire *ABCB1* gene. Reports in the literature have particularly focused on C3435T (non-coding exon 26), after an initial report on altered duodenal P-gp expression and functionality associated with the TT variant,[86] but later on the SNPs G2677T/A and C1236T have generated interest as well.

Studies within the field of psychiatry have particularly focused on treatment response to antidepressants or antipsychotics. Other studies refer to side effects and two studies pertain to the occurrence of *ABCB1* polymorphisms in mood disorders and schizophrenia. In two studies, the diagnosis of depression was found to be associated with a haplotype of 13 *ABCB1* polymorphisms.[87] In the other study, mood disorders were associated with the haplotype of 129-2677-3435 (T-A-C) and with a lower frequency of *ABCB1* alleles at -1517, -41 and -129 and a higher frequency of 2677A.[88] In this study no association was found between ABCB1 and schizophrenia.

Most studies on the genetic effects of the SNPs C3435T, G2677T/A and C1236T or ABCB1 haplotypes showed little or no effect on treatment response (table 3.4A). A

strong effect of a single transporter gene on a phenotypic response in complex disorders like MDD and schizophrenia is not amenable. A few reports on antipsychotic or antidepressant related side effects associated with polymorphisms have been published, and a few groups have communicated on the effect of *ABCB1* polymorphisms and plasma concentrations of antipsychotics or antidepressants (table 3.4B). For risperidone, displaying high substrate affinity for P-gp, only modest genetic effects were reported in side effects (weight gain) and in treatment response.[89,90]

The contradictions in observations for the major polymorphisms of ABCB1, are partly accountable to differences in methodology and to ethnic differences between study groups. In most studies the sample size is too small to draw firm conclusions.

Thus, none of the SNPs of *ABCB1*, nor any of the haplotypes, can definitely be connected to phenotypical variation, but instead some may serve as biological marker for pin pointing a disease. In the near future it is to be expected that analysis of SNP patterns in large patient cohorts with identical phenotypic features will identify SNP profiles that characterize susceptibility factors. Genome wide association studies (GWAS) are the type of study designed to identify such a genetic variation. This strategy may be best worked out in a subset of patients with severe, recurrent and early onset form of depression, since this group of patients has shown an elevated genetic contribution.[91]

Non-P-gp mediated efflux transport at the BBB

Other members of the ABC family that are expressed in the BBB, include multidrug resistance proteins (MRP1, -2, -4 and -5) (ABCC genes) and breast cancer related protein (BCRP/ABCG2 gene).[92,93] The exact location of some of the MRPs within the BBB is still debated.[93] The reasons for this uncertainty are the differences in transporter profiles between species as well as inferior antibody specificity. The predominant location of MRP1 is probably the basolateral membrane.[94] Among the non-P-gp efflux transporters MRP1 and BCRP have the highest expression in the human BBB, which however is much lower (around 20-fold) than that of P-gp.[95]

The MRPs transport anionic compounds, including sulphate, glucuronide, and glutathione (GSH) conjugates.[96] Three typical antipsychotics (thioridazine, chlorprotixene and flupentixol), all carrying a sulphur atom, were found to inhibit MRP2 *in vitro*.[96] Of the few reports on involvement of MRP1 or MRP2 in the transport of an antidepressant no significant inhibition was found.[97] BCRP appears to be involved in the efflux of antipsychotics. For the major antipsychotics Wang et al. demonstrated a similar profile of inhibition to that of P-gp,[28] albeit that for most antipsychotics at least a 5-fold higher concentration was needed to inhibit BCRP (compared to P-gp).[98] For

the antidepressants maprotiline and desipramine no significant inhibition of BCRP was reported.[99]

Concluding, the clinical relevance of the MRPs in conferring resistance to the major psychiatric drugs at the BBB is probably negligible in comparison to P-gp, given the lower substrate affinity and the much lower expression in the BBB. BCRP might exert a more significant role at the BBB and is probably more subsidiary to P-gp function than the MRPs.[99] Nevertheless, the impact of BCRP in drug disposition at the BBB *in vivo* is still uncertain.[100]

Concluding remarks and future directions

In conclusion, the role that P-gp plays in depressive and psychotic disorders, is far from clear. Most antidepressant and antipsychotic drugs have a weak to moderate affinity as a substrate for P-gp, and some may have inhibitory properties as well, which may be relevant in drug-drug interactions. P-gp may be involved in a depressive or psychotic episode as well, since evidence exists for a (temporarily) decrease in activity and expression during a neuroinflammatory event, which characterizes the pathophysiology of these disorders.

Given the importance of P-glycoprotein in drug transport at the BBB and its involvement in neuroinflammation, it is comprehensible that the protein is of great interest in the field of neuroscience. At this time, the understanding of the precise role in neuropsychiatric disorders and drug disposition is incomplete, but new developments in neuroimaging, pharmacogenetics and molecular biology are in full progress and it will be only a matter of time to accomplish this. As outlined above, an important step forward in the identification of genetic variants has been made, albeit the complex regulatory pathways involved in P-gp modulation require further considerations in pharmacogenetic studies. Studies with larger sample size will be needed to detect modest effects. Progress in PET imaging largely depends on development of new tracers that approach the unmet needs of an ideal tracer.

Table 3.1

Substrate affinity for P-glycoprotein and inhibitory properties of current antidepressants

Antidepressant	substrate properties		Inhibition compared to typical P-gp inhibitor*	references
	in vivo	in vitro		
amitriptyline	++		weak	24,25,33,45
bupropion		0	no	43
citalopram	+	0	No	25,26 31,35,101
clomipramine			no	25
desipramine			weak-moderate	24,25
doxepin	+		weak	24; 35
duloxetine			moderate	102
fluoxetine	0/+		no	25,26,31,33
fluvoxamine	+	0	weak	26,29,31,33,103
imipramine			weak-moderate	24,25
maprotiline			weak	24
mirtazapine	0		weak	25,35
nefazodone		0	good	23
nortriptyline	+		weak	25,31-33
paroxetine	+	++	moderate	29,31,101,103
reboxetine			weak	25,26
sertraline	0	+++	moderate	31,43,26
trazodone	0	0	no	23,31
trimipramine	+			34
venlafaxine	+	0	no	26,31,35,104

Legend (table 3.1-3.3)

substrate affinity *in vivo* denotes the ratio between *in vivo* brain uptake in ABCB1a -/- knock-out vs. wild type (KO-WT) mice: 0: no significant change; +: 1-3; ++: 3-10; +++:>10.

substrate affinity (*in vitro*): transport capacity compared to a prototypical P-gp substrate (mostly expressed as IC_{50} or EC_{50}, see text): 0: 0-5% ; +: 5-25%; ++: 25-75%; +++: >75%

Inhibition: good = comparable to comparator inhibitor* (at least 75%) ; moderate: 25-75% inhibition of comparator; weak: 5-25% inhibition ; no: < 5 % inhibition or non definable ; *: inhibition (*in vitro*) is related to a typical comparator inhibitor, either verapamil, ivermectin, cyclosporine, or PSC833; $^{\alpha}$ P-gp inhibition compared to PSC833 ; $^{\partial}$significant increase in lamotrigine brain/plasma ratio after administration of a P-gp inhibitor (verapamil)

Table 3.2

Substrate affinity for P-glycoprotein and inhibitory properties of current antipsychotics

antipsychotic	substrate properties		Inhibition compared to typical P-gp inhibitor*	references
	in vivo	in vitro		
amisulpiride	0	+	Weak	29
aripiprazole	++	++		39,46,105
chlorpromazine	+	+/++	Weak	25,28,31,106
clozapine	0/+	0/+	Weak	22,25,28,29,31,38,106
fluphenazine	+	++	Moderate	24,29,107
flupentixol		+	Good	29
haloperidol	0/+	0/+	weak[α]-good	25,29; 28,106,108; 24,31,37,38
olanzapine	+	++	no-weak[α]	29,106; 28,40
paliperidone	+++	++	No	28,31,36,36,37,41
perospirone			Good	109
perphenazine			Good	29
quetiapine		++	weak[α]-good	28,29,106
risperidone	+++	+++	weak-moderate	36,106
				28,36,37,41,110

Table 3.3

Substrate affinity for P-glycoprotein and inhibitory properties of mood stabilizers

mood stabilizer	substrate properties		Inhibition compared to typical P-gp inhibitor*	references
	in vivo	in vitro		
carbamazepine	0	0/+	no-weak	22,30,44,62,111-116
lamotrigine	0/+[∂]	0/+	no-weak	31, 44, 112,115-117
lithiumcarbonate				
valproic acid		0	Weak	44,112,113,116,118

Table 3.4

3.4A: treatment response to antidepressants and antipsychotics related to ABCB1 polymorphisms

Drug	DSM[a] diag-nosis	*ABCB1* variant	rs-id[b]	n cases[c]	outcome measure[d-i]	refer ences	p-value[j]
amitriptyline		G2677T/A	rs2032583	50	% change in HAMD response after 3 weeks	119	n.s.
amitriptyline, venlafaxine or citalopram	MDD	Intron 4	rs2235015	133	% change in HAMD-21 score after 4 weeks	120	0.0024
		(C/T) intron 22	rs2032583	132			0.00007
desipramine	MDD	UTR	rs17064	272	% change in HAMD-21 score after 8 weeks	87	n.s.
		haplotype block 1			remission versus non-remission after 8 weeks		0.02
paroxetine	MDD	(C/T) intron 22	rs2032583	124	% change in HAMD-21 score after 8 weeks	121	0.024
		Intron 4	rs2235015				n.s.
		(A/G) intron 21	rs2235040				0.028
	MDD	G2677T/A	rs2032583	68	% change in HAMD response after 6 weeks	122	0.01
		C3435T	rs1045642				n.s.
		C1236T	rs1128503				n.s.
		Haplotype 3435C-2677G-1236T					0.014
	MDD	G2677T/A	rs2032582	127	% change in HAMD response after 6 weeks	123	n.s.
		C3435T	rs1045642				n.s.
duloxetine	MDD	(A/C) (intron 22)	rs10280101	238	response after 5 weeks	124	n.s.
		(A/G) (intron 22)	rs7787082	237			n.s.
		(C/T) intron 22	rs2032583	238			n.s.
		(A/G) intron 21	rs2235040	239			n.s.
citalopram	MDD	C1236T	rs1128503	652	response (395) vs. non-response (257) on (different doses) after 12 weeks	125	n.s.
		G2677T	rs2032582				n.s.
		C3435T	rs1045642				n.s.

escitalopram	MDD	C1236T	rs1128503	100	Remission versus non-remission after 8 weeks	126	n.s.
		C3435T	rs1045642				0.045
		haplotype block 1 (intron26-27)					0.003
		haplotype block 2 (incl. exon13)					n.s.
fluoxetine	MDD	G1236A	rs1128503	142	% change in HAMD-21 score after 8 weeks	87	n.s.
		(T/C)	rs10276036				n.s
		(T/A)	rs2235020				n.s
		(C/G)	rs2214103				n.s.
		haplotype block 1		272	remission versus non-remission after 8 weeks		0.03
		haplotype block 2		272	remission versus non-remission after 8 weeks		0.04
fluoxetine or desipramine	MDD	haplotype block 3		272	% change in HAMD-21 score after 8 weeks		0.01
nortriptyline	MDD	C3435T	rs1045642	160	MADRAS score after 6 weeks	127	n.s.
olanzapine	S	C3435T	rs1045642	41	BPRS change after 6 weeks, related to olanzapine concentration	128	n.s.
		G2677T/A	rs2032582				0.03
		C1236T	rs1128503				0.04
risperidone	S	G2677T	rs2032582	54	> 50% improvement on PANSS score	129	n.s.
		C3435T	rs1045642	58			n.s.
	S	C1236T	rs1128503	115	% change on BPRS after 8 weeks	90	0.021
		G2677T	rs2032582				n.s.
		C3435T	rs1045642				n.s.

Table 3.4B: side effects of antidepressants and antipsychotics related to ABCB1 polymorphisms

Drug	DSM [a] diagnosis	*ABCB1* variant	rs-id [b]	n [c] cases	outcome measure [d-h]	references	p –value [h]
amitriptyline	MDD	G2677T/A	rs2032582	50	C/D ratio	[119]	n.s.
clozapine	S	C3435T	rs1045642	75	C/D ratio	[130]	0.046
		G2677T/A	rs2032582				n.s.
fluvoxamine	MDD	C3435T	rs1045642	62	higher C/D ratio in T-genotypes	[131]	0.026
olanzapine	S	C3435T	rs1045642	41	weight gain related to olanzapine concentration	[128]	n.s.
	S	C3435T	rs1045642	56	weight gain (> 7% vs < 7%)	[89]	n.s.
		G2677T/A	rs2032582				n.s.
	S	C3435T	rs1045642	122	C/D ratio (variable doses)	[132]	n.s.
		G2677T	rs2032582				n.s.
risperidone	S	C3435T	rs1045642	47	weight gain (> 7% vs < 7%)	[89]	0.015
		G2677T	rs2032582				0.031
		Haplotype 3435T-2677T					0.033
	S	G2677T	rs2032582	83	higher C/D ratio (active moiety) in TT-genotypes	[129]	0.001
		C3435T	rs1045642		higher C/D ratio (active moiety) in TT-genotypes		0.004
	S	C3435T	rs1045642	85	C/D ratio (risperidone and/or 9-OH-risperidone)	[133]	n.s.
		G2677T/A	rs2032582				n.s.

Legend (table 3.4A and 3.4B)

[a] DSM diagnosis: diagnostic statistical manual; MDD = Major depressive disorder; S= schizophrenia; [b]rs-id = reference seauence identity; [c]n = number; [d]HAMD(-21) = Hamilton Depression Rating Scale (-21 item version); [e]MADRAS = Montgomery Asberg Rating Scale; [f]BPRS = Brief Psychiatric Rating Scale; [g]CIT = citalopram ; [h] C/D = concentration-to-dose; [i]PANSS = positive and negative symptom scale ; [j]n.s. = non significant

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Chapter 4

Locally increased P-glycoprotein function in major depression

A PET study with [^{11}C]-verapamil as a probe for P-glycoprotein function in the blood-brain barrier.

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Abstract

The etiology of depressive disorder remains unknown, although genetic susceptibility and exposure to neurotoxins are currently being discussed as possible contributors to this disorder. In normal circumstances, the brain is protected against blood borne toxic influences by the blood-brain barrier, which includes the molecular efflux pump P-glycoprotein (P-gp) in the vessel wall of brain capillaries. We hypothesised that P-gp function in the blood-brain barrier is changed in patients with major depression. Positron-emission tomography was used to measure brain uptake of [^{11}C]-verapamil, which is normally expelled from the brain by P-gp. Cerebral volume of distribution (V_T) of [^{11}C]verapamil was used as measure of P-gp function. Both region of interest (ROI) analysis and voxel analysis using statistical parametric mapping (SPM2) were performed to assess regional brain P-gp function. We found that patients with a major depressive episode, using antidepressants, compared to healthy controls showed a significant decrease of [^{11}C]-verapamil uptake in different areas throughout the brain, in particular in frontal and temporal regions. The decreased [^{11}C]-verapamil-uptake correlates with an increased function of the P-gp protein and may be related to chronic use of psychotropic drugs. Our results may explain why treatment resistant depression can develop.

Introduction

A large body of evidence gathered during the past decades indicates that brain monoaminergic systems play a key role in the pathogenesis of affective disorders. However, not all symptoms of depression can be related to dysfunctions in monoaminergic systems. For instance, it is now known that stressful events paving the way to affective disorders, lead to changes in neuroplasticity, impair neurogenesis and may lead to a neuroinflammatory response in the brain.[1] As such, it has been suggested that a dysfunction of the blood-brain barrier (BBB) and blood-cerebrospinal fluid barrier may contribute to the pathophysiology of major depressive disorder. Hampel and colleagues reported that serum/cerebrospinal fluid ratios of several inflammatory proteins (used as an indirect measure of BBB function) were altered in depression.[2,3] Several endogenous substances, such as cortisol, are found in the brain parenchyma in concentrations in subjects with a major depressive disorder.

The BBB is formed by the brain capillary non-fenestrated polarized endothelial cells that have high-resistance tight junctions. Besides low passive permeability, the brain is protected from potentially harmful endogenous and exogenous substances by efflux transporter proteins, located in the brain capillary wall.
P-glycoprotein (P-gp), a product of the MDR-1 gene, now denoted as ABCB1, in humans and the MDR-1a and MDR-1b genes in mice, is a major drug efflux transporter, involved in the efflux of a wide variety of lipophilic drugs and endogenous substances.[4] Attenuation of P-gp function, for example through use of pharmacologic inhibitors, results in substantial changes in the pharmacokinetics and pharmacodynamics of various substrates.[5-7]

Nuclear medicine imaging techniques such as PET (Positron Emission Tomography) have been evaluated as non-invasive techniques for the evaluation of the BBB, allowing study of the P-gp pump.[8] Using several experimental designs, the applicability of [^{11}C]-verapamil as PET-tracer has been extensively studied[9-11] and has been found to be a suitable methodology for the in vivo assessment of P-gp functionality in humans, and allows the quantification of BBB-P-gp function in neurodegenerative diseases.[8,10-12]

The protective role of P-gp may be negatively influenced in neurodegenerative diseases. Animal models of neuroinflammation have demonstrated that P-gp is downregulated by proinflammatory cytokines.[13-15] In a recent PET study using [^{11}C]-verapamil it was found that the function of BBB P-gp was diminished in later stages of Parkinson's disease, whereas de novo patients with Parkinson showed a regional upregulation of P-gp in frontal regions.[16] In another [^{11}C]-verapamil PET study in patients with schizophrenia it was shown that P-gp function was locally increased.[17]

The authors state that P-gp induction may be a factor of importance in drug resistance in schizophrenia. P-gp function is possibly under the influence of genetic polymorphisms.[18]

To date, no detailed evidence is available for a dysfunction of the Blood-Brain Barrier through P-glycoprotein modulation in major depressive disorder. It is not known whether P-gp function or expression is altered during a depressive episode.

In this exploratory study we hypothesized that P-glycoprotein function would be altered in limbic areas (hippocampus, amygdala) as well in frontotemporal areas (incl. anterior cingulate cortex) since these areas are known to play a role in depression.[19,20]

We performed positron emission tomography (PET) brain imaging of P-gp with [^{11}C]-verapamil as radiotracer in depressed patients and healthy controls. We further hypothesized that this altered functional activity of P-gp could be connected to a genetic polymorphism of the *ABCB1* gene. To assess these hypothesized changes in P-gp function, PET brain imaging with [^{11}C]-verapamil as radiotracer was performed in depressed patients and healthy controls. The distribution volume of [^{11}C]-verapamil was used as a measure of total P-gp function.

Methods

Subjects

Fourteen patients suffering from depression were recruited and participated in the study. All underwent a Mini International Neuropsychiatric Interview, (M.I.N.I. plus 5.0.0., Dutch Version, 2000) for DSM-IV and fulfilled the criteria for a major depressive episode.[21] Inclusion criteria for subjects with a major depressive episode were 1) age 40-80 years; 2) fulfilment of the DSM-IV criteria for major depressive episode; and 3) capacity to give informed consent. The age range 40-80 years was chosen, because we expected to find larger differences at a later age in particular, since P-gp function declines with aging.[22] Moreover, the control group was also used in another study. Exclusion criteria were 1) use of known P-gp modulating agents (cardiovascular drugs, antimalarial drugs, cyclosporine A, phenothiazines, hormones (eg. tamoxifen), certain antibiotics such as cefoperazone, ceftriaxone, erythromycine);[23] 2) any somatic disease of kidney, liver, heart or brain; 3) history of traumatic brain damage; 4) electroconvulsive treatment in the past three months; 5) abnormalities at clinical (incl. neurological) and laboratory examination; 6) pregnancy. This research was approved by the Ethics Committee of the University Medical Centre Groningen, and all subjects gave written informed consent according to the Declaration of Helsinki. All patients

had a minimum score of at least 19 on the 17-item Hamilton Depression Rating Scale at the time of the PET study.[24] All patients had a physical examination and laboratory evaluation. All were in good physical health and none had meaningful laboratory abnormalities. The healthy controls as well as their first degree relatives were required to have no history of any psychiatric disease, nor in their first degree relatives. The other inclusion and exclusion criteria for healthy controls were similar to the patients. Before the scan, blood was drawn from the venous canula for genotyping. Three common MDR1 single nucleotide polymorphisms were detected using a PCR analysis.

Radiochemistry

Racemic [^{11}C]-verapamil was produced as previously described.[25] The injected radioactivity of [^{11}C]-verapamil was comparable for the control group and subjects with a major depressive episode (see Results section). Specific activity for all subjects was at least 16 GBq/μmol. Following radiotracer injection, subjects underwent a dynamic PET acquisition protocol as described previously.[12]

PET procedure

All scans were performed with the use of an ECAT EXACT HR+ position camera (Siemens/CTI, Knoxville, TN). After the radiotracer injection of [^{11}C]-verapamil serial dynamic PET scanning was done at escalating time frames and serial arterial blood sampling for [^{11}C]-verapamil took place during the scan in order to define the input function. The samples of all subjects were collected with an automated sampling system, together with 6 manually drawn samples per subject. These samples (collected at intervals of 10 minutes) were further processed to measure the radioactivity in plasma and blood. In this way the contribution of the injected activity to the signal could be calculated. No metabolite analysis was done. Images were reconstructed in brain mode using an iterative reconstruction (Ordered Subsets - Expectation Maximization) with 4 iterations and 16 subsets and a Gaussian filter of 4 mm. The scans were performed in 3D mode.

Data analysis

The PET data were analyzed with both a voxel-wise group analysis using statistical parametric mapping (SPM2) and a ROI (regions of interest)-based approach. Results from both methods were used and compared.

First, all images were stereotaxically normalized to MNI space using SPM2 and a [^{11}C]-verapamil template image that could be used from earlier studies of our group.[12,22] The resulting images were analysed with SPM2 as described below. For the ROI analysis the following ROIs, based on the literature as cited above, were selected for analysis: prefrontal cortex, anterior cingulate cortex, temporal lobes, amygdala and

hippocampus. Therefore, predefined ROIs from the Anatomical Automated Labeling package[26] were used to select the appropriate voxels and calculate the corresponding time-activity curves using in-house developed software. In addition, a whole brain ROI was manually drawn using Clinical Applications Programming Package software (CAPP; CTI/Siemens PET Systems, Knoxville, USA).

A graphical analysis according to Logan for quantification of the dynamic PET data was done with plasma data as input. The Logan plot was started at 5 minutes. With this method the distribution volume (V_T) was estimated. Because the slope (i.e.V_T effect) obtained in the graphical approach may be biased in the presence of noisy data,[27] we verified the V_T in a kinetic analysis (i.e. single tissue compartment model). The influx rate constant (K_1) and efflux k_2 were derived from this model and on all parameters (i.e. V_T, K_1 and k_2) the group means (patient group vs. control group) were compared with each other, using parametric tests. Analysis of covariance was performed in order to find relevant (clinical) predictors (age, length of present episode, number of previous episodes, severity of symptoms) of V_T or K_1. To exclude a possible confounding effect of subjects without a strict DSM-IV diagnosis (i.e. Diagnostic Statistical Manual, 4th ed.) of Major Depressive Disorder (MDD), a subanalysis was also done for the group with MDD only (n=10).

Data were then analyzed with SPM2 (Statistical Parametric Mapping, 2002). To adjust for differences in individual neuroanatomy and to improve the signal-to-noise ratio, a 12-mm full-width at half-maximum Gaussian smoothing filter was applied to all images. We first compared the groups by looking at absolute differences in V_T, using t-test and ANCOVA with cofactors (MDR1 allelic variation, age, length of present episode, number of previous trials, severity of symptoms, injected activity of [^{11}C]-verapamil). Clusters of 8 or more voxels at a threshold of $P_{FDR}<0.05$ (false discovery rate) were considered to be significant. Coordinates were transformed into Talairach space (Talairach and Tournoux, 1988) using the mni2tal-transformation (http://www.mrc-cbu.cam.ac.uk/Imaging/).

Results

The mean injected [^{11}C]-verapamil dose was not different for the two groups (control group: mean = 317 MBq, SD = 119; group with depression: mean = 279 MBq; SD = 179; t-test: t=-0.344; d.f.=24; p=0.734). Table 4.1 shows the demographic characteristics and medication use of the patient group. Twelve patients had had a previous depressive episode, three patients had a bipolar type I disorder, the current episode being depressive.

Three subjects had a depressive episode with psychotic features. One patient was excluded from the analysis because the V_T was considered an outlier since the difference to the group mean was 4.6 times the standard deviation of the group, for unknown reasons. The patient group (6 female/7 males, mean age 54.1 years ± 7.6) was compared to a sex matched comparison group (6 female and 7 male subjects; mean age 56.3 ± 14.3 years). Patients had a mean score of 25.8 (± 4.8) on the 17-item HAM-D (range 19-37). Nine patients were currently depressed for more than 12 weeks. Interestingly, 7 of this group could be considered to be treatment resistant, using criteria of Dunner).[28] Comorbid disorders included post traumatic stress disorder (PTSD) (n=1) and panic disorder (n = 1). All subjects used an antidepressant or a mood stabilizer at the time of scanning.

We determined three common MDR1 single nucleotide polymorphisms (SNP) (-129 T >C (exon 1), 2677 G> T/A (exon 21) and 3435 C> T (exon 26)) in our patient group. For the SNP 3435 on exon 26 the allele frequencies in the patient group were CC 23%; CT 38% and TT 39%. The allele frequencies of SNP 3435 in our subjects were not different from the frequency in the population.[29]

The mean brain time activity curves (after correction for weight and injected dose) for the whole brain of patient group and control group were compared and the area below the curve was calculated. No differences in the area below the curve was found. Both curves overlapped (data not shown). The V_T values of the ROI (whole brain) were calculated with both the Logan analysis and the 'single tissue compartment model', in order to verify that the V_T effect measured in the Logan method was concordant with another method. Pearson's correlation coefficient (r) was very good, 1.000 (p =0.000). The Logan curve gave an excellent fit in all cases. The V_T in ROI whole brain in the group of patients (n=13) with depression was lower at a nearly significant level (Z = -2.008; p=0.055). K_1 (whole brain ROI) showed no significant difference between the groups. (0.48 ± 0.15 (controls) vs. 0.41 ± 0.10 (patients); p = 0.15). Neither did the k_2 values (0.71 ± 0.10 (controls) vs. 0.75 ± 0.15 (patients); p=0.46). In the ANCOVA none of the cofactors (age, length of present episode, number of previous episodes, severity of symptoms) showed a significant effect on the results. Both groups were compared with a Student's t-test for the selected ROIs (prefrontal cortex, anterior cingulate cortex, temporal lobes, hippocampi, amygdala). Here we found a significantly lower V_T in the patient group for the prefrontal cortex (0.64 ± 0.20 (controls) vs. 0.44 ± 0.15 (patients); p=0.009), the temporal lobes (0.66 ± 0.21 (controls) vs. 0.44 ± 0.19 (patients); p=0.01), the anterior cingulate cortex (0.53 ± 0.20 (controls) vs. 0.34 ±0.18 (patients); p=0.016), and for amygdala (0.72 ± 0.30 (controls) vs. 0.49 ± 0.24 (patients); p=0.045) but not for hippocampus (0.55 ± 0.32 (controls) vs. 0.41 ± 0.13 (patients); p= 0.146). V_T differences in prefrontal cortex and temporal lobes were significant after Bonferroni

Table 4.1

Subject no	Treat-ment Setting	Gender	Age	AO	LPE (wk)	DSM-IV	HAMD	Medication
1	PC	M	47	22	20	BP-I, D,P	22	valproic acid 2000 mg/d (level 79 mg/l); venlafaxine 225 mg/d, risperidone 2 mg/d
2	O	F	72	67	12	MDD	20	mirtazapine 30 mg/d, temazepam 20 mg 1 dd 1; cetirizine 10 mg/d
3	PC	F	71	45	104	MDD	25	imipramine 100mg/d; lactulose 30 ml/d; esomeprazol 20 mg/d; quetiapine 200 mg/d
4	DT	F	51	27	26	BP-I,D	22	lithium carbonate 900 mg/d (level 0·88 mg/l); diclofenac 150 mg/d
5	PC	F	52	33	26	MDD,P	37	tranylcypromine 60 mg/d; olanzapine 15 mg/d
6	PC	M	60	60	8	MDD, P	26	citalopram 20 mg/d
7	PC	M	56	51	52	MDD PTSD	31	lithiumcarbonate 1000 mg/d (level 0·90 mg/L); nortriptyline 100 mg/d
8	PC	M	55	23	26	MDD, PD	25	nortriptyline 100 mg/d
9	PC	F	56	31	104	MDD	27	tolderodine 4 mg/d; temazepam 20 mg/d; cisordinol 2 mg/d; mirtazapine 30 mg/d
10	O	M	41	39	6	MDD	24	citalopram 40 mg/d; mirtazapine 30 mg/d; oxazepam 50 mg/d
11	PC	F	52	38	12	BP-I	29	valproic acid 1500 mg/d; temazepam 20 mg/d; asacol; thyrax 75 µg/d
12	PC	M	46	46	20	MDD	24	venlafaxine 150 mg/d
13	PC	M	52	44	6	MDD	30	venlafaxine 150 mg/d
14	O	F	57	56	12	MDD	19	mirtazapine 30 mg/d; citalopram 20 mg/d

Characteristics of subjects with major depressive episode

AO: Age of onset; LPE: length of present episode; O=outpatient; DT = day treatment; PC = psychiatric clinic; M = male; F = female; HAMD = Hamilton Depression Rating Scale; BP-I = Bipolar disorder type I; D = (current phase) depressive; MDD = Major Depressive Disorder, P = psychotic features; PTSD Post Traumatic Stress Disorder, PD = panic disorder

correction for multiple tests. The mean ± S.D. of V_T for each group are shown for each ROI in figure 1.

A pixel-by pixel *t* test (without scaling) comparing both groups (13 vs. 13) in SPM2, showed several clusters, mainly located in temporal and frontal regions, in which the tracer uptake (V_T) was lower in the patient group, P_{fdr} = 0.028 (see table 4.2). The largest cluster measured 52 cm^3, including predominantly temporal lobes, reaching from the precentral gyrus to cerebellum and to the parietal lobes. The other large cluster (36 cm^3) included the prefrontal cortex (see fig. 2). The clusters that reached statistical significance overlapped to a great extent with the area's found in the ROI-analysis. Length of present episode (in weeks), HAMD scores, number of previous episodes, allelic variation of MDR-1 polymorphisms 3435C>T and 2677G>T, diagnosis and administered verapamil activity as covariates in ANCOVA had no significant effect as confounder on the results.

Table 4.2

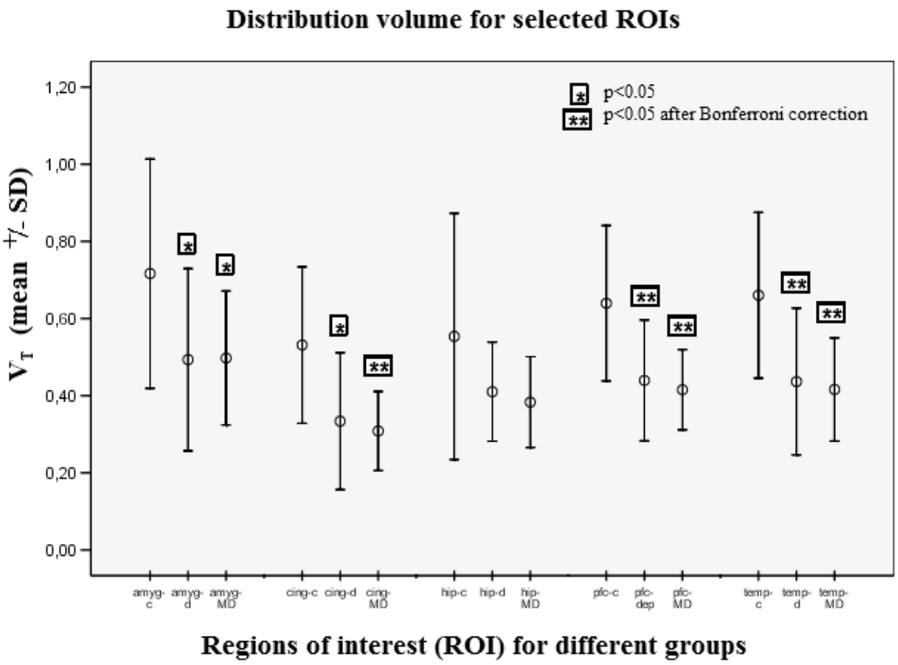
Clustersize (cm^3)	$P_{FDR-corr}$	T-value (max)	MNI coordinates	Location
52.8	0.028	3.66	-38 -2 -10	left temporal lobe, gray matter
	0.028	3.62	-12 -6 8	left thalamus, ventral anterior nc.
	0.028	3.40	28 12 -24	right temporal lobe, superior temporal gyrus
35.6	0.028	3.64	44 26 22	right frontal lobe middle frontal gyrus
		3.37	-20 46 22	left frontal lobe, superior frontal gyrus
8.0	0.028	3.37	-42 -52 -18	left cerebellum
1.5	0.028	2.83	-4 -62 4	left occipital lobe, lingual gyrus
4.3	0.028	2.81	-6 -26 48	left frontal lobe, paracentral lobule

characteristics of significant clusters in SPM2
MNI (Montreal Neurological Institute); FDR-corr = corrected false discovery rate

A subanalysis of the 10 patients with major depressive disorder (MDD) was also done. The subset was compared to a matched subset of our control group both at ROI level and in a voxelwise analysis in SPM2. The results in the voxelbased approach indicated a somewhat stronger decrease in V_T compared to the control group. A large cluster (240 cm^3, P_{FDR}=0.016, T max=4.83) was found, comprising mainly temporal lobes and frontal cortex (data not shown). The V_T in ROI whole brain was also significantly lower than in the matched control group (0.75 ± 0.25 (controls) vs. 0.55 ±0.08 (MDD); p=0.030). The results on ROI level for selected are shown in figure 4.1.

Finally, we compared the subgroup of patients with a treatment resistant depressive episode (n=7) to a matched subset of our control group. This comparison gave comparable results in both the voxelbased and pixelbased approach to the analysis of the whole group (n=13), yielding a significant decrease in V_T both on ROI level and on voxel level in the group with a treatment resistant depressive episode (data not shown).

Figure 4.1



T-test comparison of the two groups at selected regions of interest. Distribution Volume (V_T) (mean (○) and standard deviation (I) are shown in regions relevant for depression. amyg = amygdalae; hipp = hippocampus; cing = anterior cingulate cortex; pfc = prefrontal cortex; temp = temporal lobes; c = control group; d = patient group with a depressive episode (n=13); MD = subgroup (n=10) of patients with a major depressive disorder. Both patient groups showed a significant decrease in V_T in all regions but the hippocampus (* = p< 0.05, ** = p<0.05 after Bonferroni correction).

Discussion

This study shows a significantly lowered [^{11}C]-verapamil uptake (distribution volume) in prefrontal cortex and temporal lobes in patients with a major depressive episode. The results of different methods of analysis were all in accordance with each other. In functional imaging studies the prefrontal cortex and temporal lobes have often been associated with MDD.[30,31] To our knowledge this is the first study showing involvement of P-glycoprotein in medicated patients with major depressive disorder. Seven of the 13 patients studied were considered to be treatment resistant, most patients were admitted to a psychiatric hospital, indicating either a severe depressive episode or a chronic course of the illness. Although clinical parameters indicating treatment resistance or chronicity were not significant cofactors in ANCOVA, a significant decrease in V_T (in temporal and frontal areas) was found in in a subset of patients with a treatment resistant depressive disorder (compared to a matched control group).

Treatment resistance in depression may in fact be associated with increased P-gp function. Increased P-gp function may cause low uptake of antidepressants. Similar to treatment resistant epilepsy where increased expression of P-gp is associated with resistance to antiepileptics and poor prognosis,[32,33] treatment resistance may also have influenced our results.

Regional differences in V_T may reflect localized regions of higher P-gp function, which may be under the influence of a functional polymorphism of MDR1. The SNP C3435T has been associated with altered P-gp function, albeit in an intestinal cell line,[18] which does not necessarily reflect the P-gp expression in brain cells. However, the impact of genetic variations in the MDR1- gene on the course of major depressive disorder or the response on antidepressants is considered to be moderate or absent, and results are conflicting.[34-38] The frequencies of the determined polymorphism in our study group did not differ from the frequencies seen in the general population.[29,38]

Our results may also be explained by a neuroinflammatory process. There is increasing evidence for the role of cytokines in the pathogenesis of depression. Inflammation results in the release of proinflammatory cytokines, acting as neuromodulator and accounting for most of the symptoms in depression.[39,40] Experimental animal models of inflammation show that inflammation can influence P-gp expression and activity in different ways.[14,15,41] Although a decrease in function and expression of P-gp seems to be the case in acute inflammatory models, the study by Tan and colleagues shows that P-gp function was increased after the acute inflammatory phase.[42] The course of a depressive episode may be similar in such a way that in the chronic phase P-gp is upregulated.[43] Post mortem studies are warranted to confirm these hypotheses. It is desirable that further (neuro-imaging) studies are conducted to shed light on the neuro-inflammatory events in major depressive disorder.

Regional differences in V_T might be explained by an increase in atrophy in cortical brain areas in major depression. However, the most consistent findings in studies using structural MRI is reduction in hippocampal volume[44] and basal ganglia, whereas atrophy in frontal regions has been found less consistently. In contrast the most robust reduction in V_T, in our study was seen in the temporal lobes for which atrophy has not been reported. The fact that no differences in V_T were found in the hippocampus at ROI level may be due to spill-in of radioactivity of the adjacent choroid plexus, since is has been reported that there is a high accumulation of radioactivity in the choroids plexus.[45]

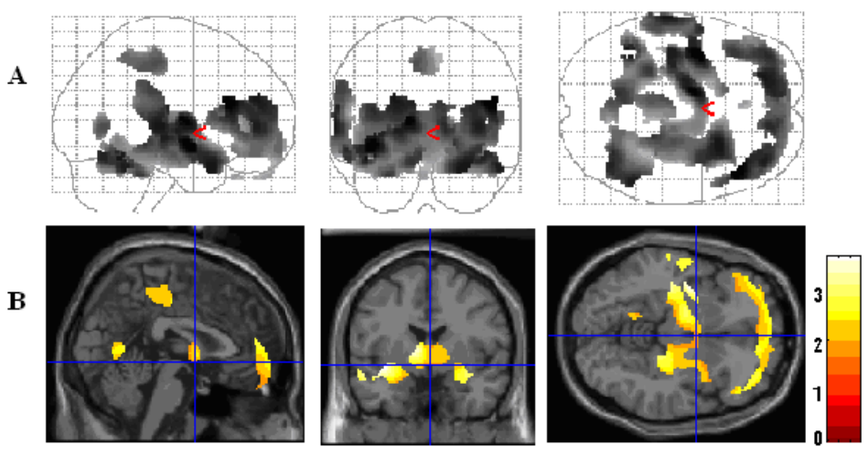
A possible limitation of the present study is the fact that patients were treated with antidepressant medication. The increase in P-gp function in our study could be caused by the use of medication. Drugs or substances that are known to enhance the P-gp expression were all excluded in our study (see table 4.1). Many of the antidepressant and antipsychotic drugs used are substrates for P-gp, but their effect on P-gp activity is probably not clinically relevant.[46-49]
Of several antidepressants and anticonvulsants it is known that they inhibit the P-gp pump in a concentration dependent manner in porcine brain cells.[48-51] In *in-vitro* studies of antupsychotics inhibition of P-gp was only seen in concentrations above therapeutically relevant plasma levels, thus suggesting that the inhibitory effect of antipsychotics may not play a role in clinical practice.[46,48] Recently, it was found that venlafaxine (used by three patients) can induce P-gp in human caco-2 cells.[52] Nevertheless, the implication of chronic use of antidepressant and/or antipsychotic drugs is however not known. It cannot be excluded that antidepressant and/or antipsychotic agents given for a sustained period lead to an up-regulation of the P-gp pump. To rule out this possibility, future studies in medication-naive patients are needed.

In addition to a possible effect on P-gp function, medication may also have influenced the metabolism of [^{11}C]-verapamil. It is known that anticonvulsants influence [^{11}C]-verapamil metabolism, probably through induction of cytochrome P450.[53] Two of our patients were on valproic acid. Leaving these 2 patients out of the analysis of difference to the results. Medication may also have influenced the free fraction of [^{11}C]-verapamil, leading to a lower V_T effect in the patient group. The influx parameter (K_1) suggested no difference between the two groups, due to a large variance. The fact that only certain areas (i.e. temporal lobes and prefrontal cortex) showed a significant decrease in V_T cannot be explained in this way.

In the present study no analysis of metabolites was performed. Although this can be seen as another limitation of the study, we assume that the total contribution of metabolites, that contribute to the PET- signal, but have no affinity for the P-gp pump, is small. Only the N-demethylated fraction (so called polar fraction) has no affinity for P-gp.[54,55] As Lubberink et al. have shown the "one tissue compartment" model gives an excellent fit of the data, irrespective of metabolite input.[54] Their data indicate that the contribution of the polar fraction to the brain signal is small. It can however not be excluded that chronic use of antipsychotic and antidepressant agents may have influenced the metabolism of [^{11}C]-verapamil, thereby reducing brain uptake of the radiotracer in patients. However, the fact that a decrease in V_T is only seen in specific areas can probably not be attributed to the metabolites of [^{11}C]-verapamil.

In summary, in our PET study using [^{11}C]-verapamil as a tracer, we have found evidence for an increased function of P-gp in patients with a major depressive episode under long term treatment conditions, which for the first time may provide an explanation for treatment resistance in patients suffering from major depressive disorder.

Figure 4.2



T-map showing a diminished Distribution Volume of [^{11}C]-verapamil in 13 patients with a depressive episode compared to the healthy control group (n = 13). The clusters that reached statistical significance in a voxel-wise *t*- test (p_{FDR} < 0 05) (in SPM2) are shown. (A) significant projections on a glass brain. (B) MRI-overlay: a large cluster located in frontal cortex, left and right temporal lobes, as well as subcortical nuclei is shown. (C) results for 10 patients with MDD compared to a matched control group (n-10).

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Chapter 5

Regional increase in P-glycoprotein function in the blood-brain-barrier of patients with chronic schizophrenia

A PET study with [^{11}C]-verapamil as a probe for P-glycoprotein function

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Abstract

P-glycoprotein (P-gp), a major efflux pump in the Blood-Brain Barrier (BBB) has a profound effect on entry of drugs, peptides and other substances into the central nervous system (CNS). The brain permeability can be negatively influenced by modulation of the transport function of P-gp. Inflammatory mediators play a role in schizophrenia, and may be able to influence the integrity of the BBB, via P-gp modulation.

We hypothesized that P-gp function in the BBB is changed in patients with schizophrenia. Positron-emission tomography was used to measure brain uptake of [^{11}C]-verapamil, which is normally extruded from the brain by P-gp. We found that patients with chronic schizophrenia using antipsychotic drugs compared to healthy controls showed a significant decrease in [^{11}C]-verapamil uptake in temporal cortex, basal ganglia and amygdalae, and a trend towards a significant decrease was seen throughout the brain. The decrease of [^{11}C]-verapamil-uptake correlates with an increased activity of the P-gp pump. Increased P-gp activity may be a factor in drug resistance in schizophrenia, induced by the use of antipsychotic agents.

Introduction

In schizophrenia, the pathological mechanism, leading to an illness affecting all domains of behavior, remains unknown. Several authors have tried to correlate the structural and functional brain abnormalities in schizophrenia with alterations in the blood-brain barrier (BBB).[1-3]

In normal circumstances, the BBB is an important factor in the protection of the brain against harmful environmental influences. The endothelial tissue forming the BBB exhibits very low permeability and expresses enzymes and efflux pumps, further limiting the passage of drugs, peptides and proteins.

There are strong indications for an impaired integrity of the BBB in schizophrenia,[3] such as increased intracerebral concentrations of the soluble intercellular adhesion molecule-1 (sICAM-1) and Very Late Antigen-4 (VLA-4) in schizophrenia have been reported.[2] Furthermore, the content of immunoglobulin and albumin is raised in cerebrospinal fluid in 30% of patients with schizophrenia. The increased permeability facilitates the penetration of inflammatory cells or the invasion of infectious pathogens, thus causing damage to the brain.[1,4]

An important element of the BBB that is increasingly recognised as a gate keeper of the brain, P-glycoprotein (P-gp), which is an efflux pump, limiting the entrance of many structurally divergent lipophilic molecules, such as peptides and many of the drugs used in psychiatry and neurology.[5,6] P-gp is encoded by the ABCB1 gene (ATP-binding cassette, sub-family B, (previously MDR-1, or Multi Drug Resistance gene). Genetic variants of ABCB1 have been proposed as potential susceptibility factors for schizophrenia and several brain diseases and as determinants of treatment response to antipsychotic drugs.[7-9] Furthermore, the protective role of the BBB may be compromised in schizophrenia due to a inflammatory or to a neurodegenerative process.[10] The activity of P-gp may be influenced bidirectionally by inflammatory reponses.[11] Moreover, P-gp function is reported to be decreased in the progression of neurodegenerative diseases.[12] However, no direct evidence is available for a dysfunction of P-gp in schizophrenia.

The activity of P-gp at the BBB can be studied in vivo by neuroimaging techniques such as PET with [^{11}C]-verapamil as PET-tracer.[13] It appears to be a suitable methodology for the in vivo assessment of P-gp functionality in humans, and allows the quantification of BBB-P-gp function in neurodegenerative diseases.[14-16] In this preliminary study we performed positron emission tomography (PET) brain imaging of P-gp with [^{11}C]-verapamil as radiotracer in schizophrenic patients and healthy controls. We hypothesized that P-glycoprotein function would be altered in dorsolateral prefrontal cortex, amygdala, thalamic nuclei, temporal areas as well basal ganglia since these areas are known to play a role in schizophrenia.[17-20] We further hypothesized that

a change in P-gp expression would be associated with a genetic polymorphism of the ABCB1 gene.

Materials and methods

Subjects

Ten patients suffering from schizophrenia, paranoid type, were recruited and participated in the study. All underwent a Mini International Neuropsychiatric Interview (M.I.N.I. plus 5.0.0., Dutch Version, 2000) for DSM-IV and fulfilled the criteria for schizophrenia, paranoid type.[21] Inclusion criteria for subjects with schizophrenia were 1) age 18-65 years; 2) fulfilment of the DSM-IV criteria for schizophrenia; and 3) capacity to give informed consent. Exclusion criteria were 1) use of known P-gp modulating agents (cardiovascular drugs, antimalarial drugs, cyclosporine A, phenothiazines, hormones (eg. tamoxifen), certain antibiotics such as cefoperazone, ceftriaxone, erythromycine);[22] 2) any somatic disease of kidney, liver, heart or brain; 3) history of traumatic brain damage; 4) electroconvulsive treatment in the past three months; 5) abnormalities at clinical (incl. neurological) and laboratory examination; 6) pregnancy. This research was approved by the Ethics Committee of the University Medical Centre Groningen, and all subjects gave written informed consent according to the Declaration of Helsinki.

For all patients a PANSS (Positive And Negative Symptom Scale) was done at the time of the PET study.[23] All were in good physical health and none had meaningful laboratory abnormalities. The inclusion and exclusion criteria for healthy controls were similar. In addition, the healthy controls as well as their first degree relatives were required to have no history of any psychiatric disease, nor in their first degree relatives. Before the scan, blood was drawn from the venous canula for genotyping. Three common ABCB1 single nucleotide polymorphisms (T129C, G2677T/A and C3435T) were detected using a PCR-RFLP-method (polymeric chain reaction - restriction fragment length polymorphism).

Radiochemistry

Racemic [^{11}C]verapamil was produced as previously described.[24] The injected radioactivity of [^{11}C]-verapamil did not differ between control subjects (mean = 382 MBq; SD = 20) and subjects with schizophrenia (mean 385 MBq; SD = 76). In all cases, specific activity was at least 16 Gbq/µmol.

PET procedure

We used an ECAT EXACT HR+ position camera (Siemens/CTI, Knoxville, TN). After the radiotracer injection of [^{11}C]-verapamil serial dynamic PET scanning was

performed at escalating time frames over a period of 60 minutes. Continuous arterial blood sampling to measure the activity of the radiotracer took place during the scan in order to define the input function. Manual samples (collected at intervals of 10 minutes) were collected to calibrate the plasma and blood time-activity data. No metabolite analysis was done. Images were reconstructed in brain mode using an iterative reconstruction (Ordered Subsets - Expectation Maximization) with 4 iterations and 16 subsets and a Gaussian filter of 4 mm. The scans were performed in 3D mode. Regions of interest were traced using an computerized brain atlas (Talairach Daemon) (The Research Image Center UTHSCSA, University of Texas Laboratory, USA).

Data analysis

Image visualization and region of interest (ROI) analyses were performed using Clinical Applications Programming Package software (CAPP; CTI/Siemens PET Systems, Knoxville, TN). First, all images were transformed into a stereotaxic standard space. This normalizing spatial transformation matches each scan to the [^{11}C]-verapamil template that was available from an earlier study of our group.[15] Stereotaxic normalization of PET images allows comparison of scan data in identical pixels across different subjects and scans.

The data were analyzed in three ways. First the data for the whole brain were compared between patients and controls. Then a-priori defined brain regions were tested with SPM2 and appropriate small volume correction. Finally, a hypothesis generating test was performed on all voxels using SPM2 and FDR correction. In all cases the Volume of Distribution (V_T) was used as a measure for P-gp function.

The multilinear implementation approach (MA1)[25] of the Logan plot[26] was used to determine V_T with minimal bias. For the whole brain ROI analyses we additionally verified V_T in a kinetic analysis (i.e. one-tissue compartment model). Finally, using the one-tissue compartment model, the whole brain influx (K_1) was calculated also. V_T was calculated and its difference between patient group and control group was compared with t-tests and ANCOVA (analysis of covariance) with age as cofactor. Parameters in the patient group (different scores on PANSS scale, antipsychotic dose in Haloperidol equivalents (mg), number of previous episodes) were also considered as predictors in a regression analysis, or their correlation with V_T was calculated.

To test the a-priori defined brain regions, the V_T images (based on the whole brain time-activity curve) were then analyzed with SPM2 (Statistical Parametric Mapping, 2002). Based on the literature as cited above, we selected the following regions: prefrontal cortex, basal ganglia, temporal cortex, amygdalae and thalamic nuclei. These regions were defined using a single mask image encompassing all selected brain regions. Then small volume correction was applied to correct for the multiple comparison in the selected volume only. No scaling or global normalisation was

performed to test absolute differences in V_T. To adjust for differences in individual neuroanatomy and to improve the signal-to-noise ratio, a 12-mm full-width at half-maximum Gaussian smoothing filter was applied to all images. The group comparison was made after age correction (ANCOVA). Using the PET model option 'single subjects, covariates only', measurement of a potential confound (number of previous episodes, antipsychotic dose (Haldol equivalents), PANSS score could also be determined.

Finally, hypothesis generating tests were performed in SPM2. The same contrasts as mentioned above tested the main effect between the patient group and the healthy control group.

Results

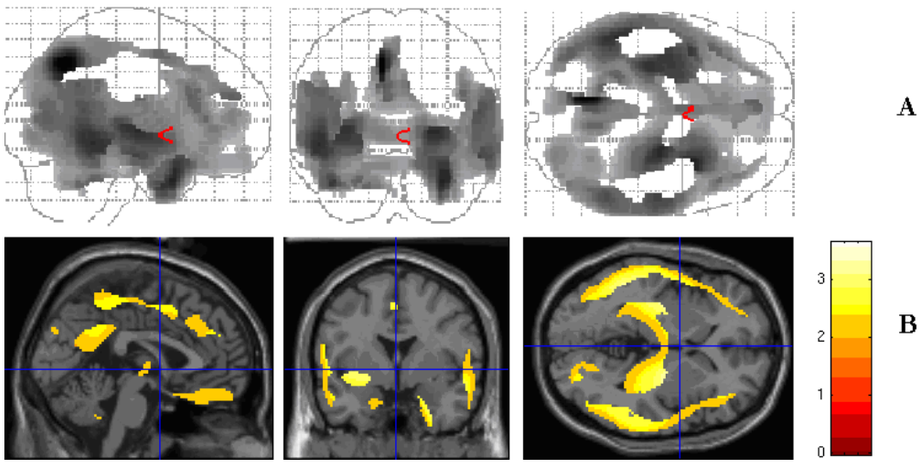
The patient group (10 males, mean age 43.9 years ± 16.9) was compared to a sex and age matched comparison group (mean age 43.0 ± 17.6 years). On the PANSS patients had a mean score of 12.9 (± 2.6) for positive symptoms 16.4 (±7.9) for negative symptoms and 24 (± 6.2) for general symptoms. All subjects had suffered three or more psychotic episodes, the last episode within 6 months prior to the PET-scan. None had a comorbid psychiatric or somatic disorder. All subjects used antipsychotic medication at the time of scanning. Table 5.1 shows the demographic characteristics and medication use of the patient group.

The mean brain time activity curves (TAC) (after correction for weight and injected dose) at brain ROI level of both groups were almost equal, the mean area under the curve (AUC) was not significantly lower for schizophrenia than for the control group. The Logan curves of all subjects gave an excellent fit of the observed data. Correlation of calculated V_T between the Logan method and the one tissue compartment model was excellent: Pearson's coefficient was 0.906 ($p<0.01$). The V_T over the whole brain was lower in the patient group (0.47 ± 0.15) than in the control group (0.67 ± 0.26), although not significantly ($t = 2.095$; $df = 18$; $p = 0.051$); K_1 was equal between the groups (controls: 0.046 ± 0.014; patients: 0.052 ± 0.023; $p = 0.47$). K_2 showed large variation in the patient group (0.17 ± 0.23) (vs. controls: 0.07 ± 0.0071) and appeared not to be equally distributed (Levene's test: $F = 5.82$; $p = 0.027$). In a nonparametric test k_2 was significantly higher in the patient group (Kolmogorov-Smirnov $Z = 1.565$; $p = 0.015$). None of the parameters (i.e. Haldol equivalents, number of previous episodes, PANSS scores) correlated with V_T or K_1 in the patient group, neither a significant effect in a linear regression model (ANOVA) was found. Additionally, we corrected the data for age (with ANCOVA), since the age variation in the sample was large. In the age

corrected model, with V_T as dependent variable, the significant trend was lost (F=2.25; p=0.136), age explained 21% of the variance in the sample. No correlation between age and controls (Pearson ρ=0.05; p=0.89) or patient group (ρ=-0.35; p=0.32) was found, however.

In SPM2 the a priori hypothesis was performed with ANCOVA (with age as cofactor) for the selected areas (temporal cortex, prefrontal cortex, amygdala, basal ganglia and thalami). Significant clusters, showing a lower V_T in the patient group, were found in the temporal cortex (P_{FDR} = 0.032), basal ganglia (P_{FDR} = 0.019), amygdala (P_{FDR} = 0.028), but not in thalamus (P_{FDR} = 0.108), and prefrontal cortex (P_{FDR} = 0.076). More details are shown in table 5.2. No significant effect of the cofactors (PANSS, number of previous episodes, antipsychotic dose) were found in SPM2 (option 'single subject, covariates only'). A hypothesis generating analysis (ANCOVA, age as cofactor) comparing both groups showed 1 large cluster and two clusters of modest size, with a significant trend at voxel level (P_{FDR} = 0.094), clusters involving many areas throughout the brain, in particular the temporal lobes, limbic lobes (right temporal lobe, 25.3 cm^3 and left temporal lobe 17.2 cm^3), the left parietal lobe (10.2 cm^3) basal ganglia, and to less extent smaller areas in the frontal lobes (see figures 1a and 1b). The areas represent a (trend to a significant) decrease in V_T in the group of patients with schizophrenia.

Figure 5.1



T-map showing a diminished a trend towards a decreased Distribution Volume of [^{11}C]-verapamil in 10 patients with schizophrenia, a depressive episode compared to the healthy control group (n = 13). compared to 10 healthy controls in sagital, coronal and axial directions. The clusters of voxels at P_{FDR} = 0.09 (in SPM2) are shown.
(A): projections on a glass brain
(B) MRI-overlay: clusters located in (fronto)temporal and parietal lobes, as well as subcortical nuclei are shown.

Table 5.1

Subject No	Age	Age of onset	NE	Rem. (months)	PANSS (P,N,G)	**Medication**
1	44	19	>5	3	14,20,38	Ris IM 50,Cloz 200, Parox 20
2	45	23	>5	12	16,9,24	Ris 2
3	40	21	>5	4	11,21,20	Cloz 300, Tem 20
4	35	24	5	6	10,6,19	Ris 6
5	40	25	>5	10	12,13,20	Cloz 300
6	23	18	4	8	13,13,21	Olanz 20
7	31	22	>5	3	16,28,22	Olanz 10 Cital 20
8	34	19	5	9	15,27,30	Ris 4, Parox 20
9	34	30	3	6	9,11,21	Olanz 20 Zuclo 20

Characteristics of subjects with schizophrenia

NE = Number of previous Episodes; Rem. (months): number of months in remission since last psychotic episode; PANSS = Positive and Negative Symptoms Scale (Sheehan et al., 1998); P,N,G = scores on symptom scales for Positive, Negative, General symptoms; Medication: oral dosis in mg per day. Cloz = Clozapine; Hal = Haloperidole; Ris = Risperidone; Parox = Paroxetine; Cital = Citalopram; Tem = Temazepam; Zuclo = Zuclopentixol; Ris IM = Risperidone Intramuscular (dosis in mg per 2 weeks)

Table 5.2

Selected region	**search volume(cm^3)**	**T-value (max)**	**P_{FDR}**
Temporal cortex	40.7	2.76	0.032
Thalamus	12.5	2.59	0.108
Basal ganglia	4.6	2.72	0.019
amygdalae	1.4	2.38	0.028
PFC	32.1	3.04	0.076

characteristics of most significant clusters of the selected volumes of interest in SPM2

PFC = prefrontal cortex; P_{FDR}= (false discovery rate) corrected p-value

Genetic Polymorphisms

Three common ABCB1 single nucleotide polymorphisms (SNP) (-129 T >C (exon 1), 2677 G> T/A (exon 21) and 3435 C> T (exon 26)) were determined in the patient group. For the SNP 3435 on exon 26 the allele frequencies in the patient group were CC 33%; CT 44% and TT 22%. For the SNP 2677 allele frequencies were 33% GT, 44% GG, 22 % TT. The frequencies of the combination of 2677GG/3435CC was 33%. The allele frequencies of the SNPs 3435 and 2677 in our subjects were not different from the frequency in the population.[27,28] Of SNP 129T>C the allele frequency was found to be TT in all cases (n=10), compared to a 96% frequency in the population (n=206).[28]

Discussion

This study shows regionally decreased [^{11}C]-verapamil uptake in temporal cortex, amygdalae and basal ganglia in patients with schizophrenia compared to healthy controls. Our study group consisted of 10 patients with chronic paranoid schizophrenia, all having suffered at least three psychotic episodes with residual symptoms. All used antipsychotic drugs. The results of different methods of analysis were in accordance with each other, showing a trend towards a significant decrease in V_T in patients with schizophrenia, which is interpreted as an increase in P-gp function, since differences in influx were not found. Moreover, a higher efflux was found at whole brain ROI level. To date, this is the first time evidence has been obtained explaining a relationship between a functional change of the efflux pump in medicated patients with schizophrenia.

Our findings may be of great value since an increase in P-gp function may be relevant for the clinical course in schizophrenia. Increased P-gp function causes low uptake of antipsychotic agents and may thus be associated with poor treatment outcome. In oncology P-gp upregulation is coupled to tumor resistance to chemotherapy and poor prognosis.[29] An increase of P-gp function in the BBB may be correlated with a more chronic course of schizophrenia. Our results may at least partially explain why often lower antipsychotic doses suffice in first episode patients. Abnormalities in the amygdala, basal ganglia and temporal cortex are consistent with available evidence, that suggests that the pathological process in schizophrenia affects many areas.[18] An open question is how and why P-gp function is regionally increased in schizophrenia, and what the treatment consequences can be of a regionally increased P-gp function.

A possible association with an increased expression or activity of (cerebral) P-gp in schizophrenic patients may be a functional polymorphism of ABCB1. The frequencies of the determined polymorphisms in our study group did however not differ from the frequencies seen in the general population,[28] although no definitive conclusions can be

drawn due to the small sample size. There are reports of great variation in the expression of P-gp,[30,31] but P-gp expression does not necessarily reflect activity.[32] The best studied SNP is C3435T and although it has been associated with altered P-gp function,[33] the results of different studies have been discordant.[34] It has been suggested that ABCB1 haplotypes should be analyzed in stead of SNPs.[8,34] However, the impact of genetic variations in the ABCB1 gene on pharmacokinetics and pharmacodynamics of P-gp substrates is considered to be moderate.[8,35]

The local P-gp increase found in our study may also be explained by a neuroinflammatory or a neurodegenerative process. Studies that focus on cytokine production in schizophrenia patients show that pro-inflammatory cytokines are actively involved,[36,37] and models of acute neuro-inflammation in cell lines show that several pro-inflammatory cytokines can reversibly inhibit P-gp function and its expression.[32,38,39] However, P-gp-activity and expression appears to be increased after the acute phase.[11,40] TNF appears to exert both an acute pro-inflammatory as well as a neuroprotective effect through different receptor subtypes.[41,42] A functional change in P-gp activity may also fit in a neurodegenerative hypothesis of schizophrenia, with long-term alterations in blood–brain barrier permeability after inflammatory events in early life.[10] Tan *et al.* demonstrated an up-regulation of P-gp in a human BBB model following exposure to activated T-lymphocytes.[43]

Another explanation for the increase in P-gp function in our study could be the use of medication that induces P-gp-function. All medication known to induce P-gp activity was excluded in our study.[22] All of the patients used antipsychotic agents and three used an antidepressant as well (see table 5.1). Most of the psychotropic drugs used are known substrates for the P-gp pump.[44-47] On the short term these drugs have shown to possess weak inhibitory effect on P-gp activity, which is probably clinically irrelevant.[44,46] Although not used by any of the patients, in a [^{11}C]-verapamil PET study we recently showed that P-gp function at the BBB is increased by chronic administration of venlafaxine.[48] The use of medication is a limitation of the study. Besides a possible effect on P-gp function, medication may also have influenced the metabolism of [^{11}C]-verapamil. Anticonvulsives for example are known to influence [^{11}C]-verapamil metabolism, probably through induction of p450 cytochromes.[49] Of antipsychotic agents this effect is however not known. Medication might also have influenced the free fraction of [^{11}C]-verapamil, consequently leading to a lower effect in the patient group. On the other hand, the influx parameter K_1 was not different between the two groups, indicating a comparable influx between the groups. Besides, the fact that only certain areas show a decrease in radio tracer uptake cannot be explained in this way.

In our study no analysis of metabolites was done. We assume that the contribution of radioactive metabolites of [^{11}C]-verapamil to the PET-signal, that has no affinity for the P-gp pump is small. Only the N-demethylated fraction (so called polar fraction) has no affinity for P-gp.[50,51] The data of Lubberink c.s. indicate that the contribution of the polar fraction to the brain signal is small. It is however possible that chronic use of antipsychotic and antidepressant agents has had influence on metabolism of [^{11}C]-verapamil, thereby reducing brain uptake of the radiotracer in patients. The fact that K_1 showed no differences between the groups and the fact that differences are only seen in specific areas militate against the role of metabolites on the PET data.

In summary, in this [^{11}C]-verapamil-PET study evidence was found for local increase in P-gp activity in medicated schizophrenia patients. Our results shed new light on the pharmacokinetics of antipsychotic drugs in schizophrenia and offers an explanation for the occurrence of drug resistance in schizophrenia. The small study group may limit the evidential value of our findings. Future studies that focus on the role of P-gp in different phases of the disease in a larger study group without the confounding influence of medication are warranted.

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Chapter 6

ABCB1 gene variants may indicate susceptibility for major depressive disorder

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Abstract

Background: P-glycoprotein, an ATP-driven efflux pump in the blood-brain barrier, has a major impact on the delivery of antidepressant drugs in the brain. Genetic variants in the gene *ABCB1* encoding for P-glycoprotein have inconsistently been associated with adverse effects. In order to resolve these inconsistencies, we conducted a study in a large cohort of patients with major depressive disorder with the aim to unravel the association of *ABCB1* variants with adverse effects of antidepressants and in particular with SSRIs, which display affinity as substrate for P-glycoprotein.
Method: The Netherlands Study of Depression and Anxiety (NESDA) study was used as a clinical sample. For 424 patients data were available on drug use, side effects. We selected six *ABCB1* gene variants (1236T>C, 2677G>T/A, 3435T>C, rs2032583, rs2235040, rs2235015) and analyzed them for association with adverse drug effects using multinomial regression analysis for both single variants and haplotypes.
Results: Number of SSRI-related adverse drug effects was significantly associated with rs2032583C (p=0.002) and rs2235040A (p=0.001) and a haplotype containing the six variants (p=0.002). Moreover, both anticholinergic (e.g dry mouth, constipation, restlessness) and serotonergic effects (sleeplessness, gastrointestinal complaints, sexual effects, twitching) were significantly predicted by these variants and haplotype (p=0.002 to 0.011).
Conclusion: Adverse drug effects with SSRI treatment, in particular anticholinergic and serotonergic effects, are predicted by common polymorphisms of the *ABCB1* gene.

Introduction

One of the major problems with pharmacotherapy of major depressive disorder (MDD) is the lack of predictive clinical variables in the occurrence of unpredictable adverse drug reactions, often leading to a prolonged treatment course. An important aspect in this regard is the ability of a drug to pass the blood-brain barrier.
P-glycoprotein (P-gp) is an ATP-driven efflux pump, predominantly located at the luminal side of the brain capillaries.[1] P-gp has an unusually broad capacity to recognize and expel hundreds of molecules from the brain, including many antidepressant drugs.[2] The processes involved often exhibit considerable interindividual variability, which may be genetically determined. P-gp is a member of the ATP-binding cassette (ABC) superfamily and is encoded by the *ABCB1* gene, formerly denoted as *MDR1* (multi-drug resistance gene 1).

Animal studies have shown that penetration of several antidepressants depends on the functionality of P-gp at the blood-brain barrier.[3-6] By affecting plasma levels and intracerebral concentration, altered P-glycoprotein function or expression may cause adverse drug effects and ultimately influence therapeutic response.
Most studies that analyzed the relation of *ABCB1* gene variants with antidepressants have focused on treatment response, with contradictory results.[7-13] Analysis of antidepressants related adverse drug effects in relation to *ABCB1* polymorphisms has not been studied extensively so far. Of the three studies to date, one study focused on a single drug effect (postural hypotension in nortriptyline use (n= 8) in a relatively small sample (n = 78).[14] In another small sample study (n=55) antidepressant-induced switch to mania was studied in 26 users of various antidepressants, without taking account of the differential affinity of antidepressants for the P-gp pump.[15] Peters *et al.* used a large sample (STAR*D, n=565) of MDD patients using citalopram and found no association between 3 single nucleotide polymorphisms (SNPs) (3435C>T, 2677G>T or 1236C>T) and 'tolerance' to citalopram. 'Tolerance' was considered when subjects continued with citalopram until the end of study period while patients who left the study at any time due to side effects, were defined as 'intolerant'.

Conflicting results may result from small sample sizes and thus limited statistical power. The present study uses data from a Dutch cohort of 424 depressed patients using selective serotonin reuptake inhibitor (SSRI), and examines whether related adverse side effects are associated with *ABCB1* gene polymorphisms.

Methods

NESDA sample

NESDA is a longitudinal, naturalistic cohort study examining the course and consequences of depressive and anxiety disorders. The collection and genotyping of the NESDA sample has been described elsewhere.[16] In brief, 2981 participants were recruited through mental health care organizations (n=807), the community setting (n=564) and primary care (n=1610). They were assessed using the Composite Interview Diagnostic Instrument (CIDI) to diagnose depressive and anxiety disorders according to the Diagnostic and Statistical Manual of Mental Disorders – Fourth Edition (DSM-IV-TR). The interview also provided information on age of onset, number of MDD episodes, and specific symptoms of depression. At baseline, detailed assessment was made of psychopathology using different inventories and scales. Many other data were collected including sociodemographic data and health indicators.

At the two-year follow up assessment phenotypic data concerning course of the current or previous depressive episodes were gathered. Medication use was assessed based on drug container inspection of drugs used and coded using the Anatomical Therapeutic Chemical (ATC) classification.[17] Use of antidepressants was considered when taken at least 50% of the time and included selective serotonin reuptake inhibitors (ATC-code N06AB). Antidepressants were categorized into SSRIs with known substrate affinity for P-glycoprotein (i.e. citalopram, paroxetine, venlafaxine, fluvoxamine, sertrailine). All other non-SSRI antidepressants were left out of the analysis (i.e. tricyclic antidepressants, St. John's Wort, lithium, MAO inhibitors) as well as SSRIs without affinity for P-gp, or without compelling evidence for the opposite (i.e. fluoxetine, bupropion, nefazodone, trazodone, mirtazapine, duloxetine,).[2,4-6,18-20] Subjects using co-medication with a strong modulatory effect on P-gp function, such as calcium blocking agents, protease inhibitors, cardiac glycosides, ketoconazole, methotrexate, quinidine,[21,22] were excluded from the analysis.

Adverse drug effects

The questionnaire on "side effect of psychotropic medication" provided a self-reported inventory of psychotropic medication (type, frequency, dosage, duration), treatment response and adverse side effects data. Adverse drug reactions (ADRs) were measured at the 1-year and 2-year follow-up. ADRs were reported as 'present' or 'absent'. The ASEC-12 self report questionnaire, a short version of the Antidepressant Side Effect Checklist, was constructed for NESDA. Recently, the ASEC-21 has been validated against the UKU Side Effect Rating Scale, a semi-structured interview by a health professional.[23,24] The ASEC-12 specifically asks for the presence of 12 ADRs, which have been related to antidepressants: sleeplessness, sleepiness during day time, restlessness, twitching/muscle cramps, dry mouth, transpiration, sexual side effects,

nausea, constipation, diarrhea, weight gain and dizziness). In addition, participants were asked to add other side effects, not covered in the inventory. Side effects were also grouped and defined as either 'anticholinergic' (i.e. restlessness, constipation, dry mouth), 'serotonergic' (i.e. diarrhea, sexual side effects, twitching, nausea, sleeplessness) or 'histaminergic' (sleepiness during the day, weight gain).
Antidepressants were categorized into SSRIs with known substrate affinity for P-glycoprotein (i.e. citalopram, paroxetine, venlafaxine, fluvoxamine, sertraline). All other non-SSRI antidepressants were left out of the analysis (i.e. tricyclic antidepressants, St. John's Wort, lithium, MAO inhibitors) as well as SSRI's without affinity for P-gp, or without compelling evidence for the opposite (i.e. fluoxetine, bupropion, nefazodone, trazodone, mirtazapine, duloxetine).[2,4-6,18-20] Sertraline was categorized as a SSRI with P-gp affinity based on high *in vitro* affinity.[20]

SNP selection and genotyping

A selection of six single nucleotide polymorphisms (SNPs) was made on the basis of positive reports in the existing literature[8,10,25,26]. This included the three common coding SNPs 3435C>T (rs1045642), 2677G>T/A (rs2032582) and 1236T>C (rs1128503) and three non-coding SNPs (rs2235015, rs2032583 and rs2235040) that were associated with treatment response to antidepressants.[11,12] The genotypic data were obtained from the genome-wide association study.[27] These data were subjected to rigorous quality control criteria.[28] None of the six SNPs was directly genotyped, but estimated genotype probabilities were obtained through imputation.[29] The quality of imputation was good (p=0.93) for 3435C>T and excellent (p=0.99-1.00) for the other SNPs. More details are presented in table 6.1. '

Table 6.1

SNP	reference sequence number	position*	coded/ other allele	coded allele frequency	Imputation quality	role
3435	rs1045642	86976581	C/T	0.444	0.93	coding exon 27
2677	rs2032582	86998554	G/T	0.577	0.99	coding exon 22
1236	rs1128503	87017537	C/T	0.579	1.00	coding exon 13
	rs2235015	87037500	G/T	0.793	0.99	Intron 5
	rs2235040	87003686	A/G	0.134	0.99	intron boundary exon 21
	rs2032583	86998497	C/T	0.134	1.00	intron 22

Major characteristics of all single nucleotide polymorphisms
* Position from NCBI Genome build 36, release 22

Covariates and exclusion criteria

We excluded 16 participants who used medication with a strong inhibitory effect on P-gp function (calcium blocking agents, protease inhibitors, cardiac glycosides,

ketoconazole, methotrexate, quinidine)[21,22] were excluded from the analysis. The group of subjects using statin drugs (e.g. simvastatin, ATC codes: C10AA01-C10BA02)) (n=42) was considered large enough to include statin use in all analyses as a covariate. The variables 'age' and 'gender' were also included in each analysis as covariates, since these variables have an impact on the side effect profile of antidepressants.[30,31]

Statistical analysis

Hardy–Weinberg equilibrium was evaluated by χ^2 statistics. No SNPs were found to violate Hardy-Weinberg equilibrium using a Bonferroni-corrected threshold. Multinomial logistic regression analysis was conducted for SSRI-related adverse effects divided in three groups, namely 0, 1-3 and 4-12 adverse effects, with allele dosages of the SNPs as independent variable.

A multinomial analysis was also done for aggregated groups of adverse drug effects (i.e. serotonergic, anticholinergic, histaminergic (see above)). Likewise, these variables were recoded into multinomial variables (0, 1, or 2 or more adverse effects). Furthermore, all adverse drug effects were tested as dichotomous variable (absent or present) in a logistic regression analysis, on possible associations with the polymorphisms that had shown an effect in the multinomial analysis. The analyses were carried out for the group of SSRI users (SSRIs with affinity for P-gp), as well as for paroxetine and citalopram separately, since these groups were large enough for analysis. Follow-up analysis with only the two SNPs that had shown an association in the multinomial analysis, were carried out for the three aggregated groups (i.e. anticholinergic, serotonergic and histaminergic) and 12 separate adverse drug effects. The (multinomial) regression analyses for the variables 'histaminergic' and 'serotonergic' was done with dichotomous variables, because too few cases (4 and 6) remained in the 2+ group, making the validity of the model uncertain.

Frequencies of the haplotypes containing the six SNPs were estimated using the following randomization procedure. For each individual and each SNP a genotype was assigned randomly five times from its imputed genotype probability distribution. Next for each randomized set the software package PHASE was used to estimate haplotype probabilities for each individual.[32,33] The haplotype probabilities were aggregated and frequencies of the 64 possible haplotypes were calculated.[32,33] For each of the four haplotypes with a frequency of >10%, haplotype dosage (i.e. the estimated number of haplotypes in an individual) was determined for every individual using the estimated haplotype probabilities like is done for Haplotype Trend Regression analysis.[34] Haplotype analysis was performed using multinomial or logistic regression analysis similar to the single SNP analyses described earlier. In addition, we used these frequencies to estimate linkage disequilibrium (LD) of each pair of SNPs. Given the

strong LD for two SNP pairs (2677G>T/A-1236T>C: r^2 = 0.88 and rs2235040-rs2032583: r^2 = 0.98), the significance threshold was set at 0.0125 as a result of a Bonferroni correction for multiple testing. Statistical analyses were conducted using SPSS software version 16.0 (SPSS Inc., Chicago, Illinois, USA) and Microsoft Excel® 2003 (Microsoft corporation, Redmond, Washington, USA).

Results

The sample consisted of 504 SSRI users, of which 347 were current users and 157 had used an SSRI before. 16 were excluded because of use of comedication with a strong inhibitory effect on P-gp function. For 64 cases genotype or interview data were incomplete, and these were also excluded from the analysis. The remaining 424 cases were used for analysis. Details of the study sample are given in table 6.2.

Adverse side effects

For SSRI use, the most reported side effects were sexual dysfunction (19.0%), dry mouth (18.8%), sleepiness during the day (18.8%), weight gain (17.7%) and profuse sweating (14.5%). Gastrointestinal side effects were relatively infrequent: diarrhoea (3.8%), nausea (10.1%) and constipation (8.9%). Other reported side effects in the inventory were dizziness (14.1%), sleeplessness (7.5%), restlessness (6.5%) and muscle spasms or twitching (8.7%). For the SSRI users (n = 424), two SNPs were significantly associated with the number of side effects (rs2235040A: p = 0.002; OR= 2.32; and rs2032583C: p = 0.001, OR = 2.41), see table 6.3. Sex and age had no significant effect as covariate in these analyses. Statin use had no significant effect in any of the analyses.

Of the three groups of aggregated side effects, 'anticholinergic' 'serotonergic' and 'histaminergic', the two SNPs were also significantly associated with both 'multiple' anticholinergic effects: rs2235040A (p = 0.010; OR = 1.92), rs2032583C (p = 0.010; OR = 1.92) and 'few' serotonergic effects: rs2235040A (p = 0.005; OR = 1.85), rs2032583C (p = 0.002; OR = 1.96). Male gender was strongly associated (OR = 1.96; p = 0.004) with more 'serotonergic' effects (containing the variable 'sexual effects'), but not with anticholinergic (p = 0.053). No association was found with 'multiple' serotonergic effects, due to the fact that this group contained only 16 subjects. No associations were found with 'histaminergic' effects.

The two SNPs were also evaluated as predictors of each single side effect in adjusted logistic regression analyses. The variable 'sleepless' was strongly associated with

rs2235040A (p = 0.0003; OR = 2.93) and rs2032583C (p = 0.0003; OR = 2.95). The two SNPs were (after correction for multiple tests) not significantly associated with the occurrence of 'constipation' (rs2235040A: p = 0.047; OR = 1.85 and rs2032583C: p = 0.049: OR= 1.84). Male gender was significantly associated with the occurrence of SSRI-related sexual side effects (p = 0.0003; OR = 2.63). There was a non significant association for use of statins (p = 0.064) for the variable 'weight gain'.

The two mostly used SSRIs in this study, paroxetine (n=151) and citalopram (n = 106), were separately tested for possible gene related side effects. For paroxetine, we found nominal significant associations between the two SNPs and 'multiple' anticholinergic effects (for rs2235040A: OR= 3.22; p = 0.034 and for rs2032583: OR = 3.28; p = 0.031), but these did not withstand multiple testing correction. No effects were found for citalopram (data not shown).

Table 6.2

Subjects using an SSRI (with P-gp affinity) (n)	**424**
sex (M:F)	133:291
age (mean ± SD)	41.0 ±11.8
citalopram (n)	106
average dose (mg ± SD)	20.3 ± 8.53
paroxetine (n)	151
average dose (mg ± SD)	20.2 ± 5.17
venlafaxine (n)	87
average dose (mg ± SD)	107.0 ± 63.7
fluvoxamine (n)	42
average dose (mg ± SD)	69.7 ± 24.8
sertraline (n)	38
average dose (mg ± SD)	64.3 ± 23.0

Basic characteristics of the study sample

Haplotype analysis

Of the 64 haplotypes, covering the six SNPs used in the analysis (3435C>T-2677G>T/A-1236T>C-rs2235015-rs2032583-rs2235040), we analyzed the four with a frequency higher than 10%: T-T-T-G-T-G (39%), T-G-C-G-T-G 14%, C-G-C-T-C-A, 13%, and C-G-C-G-T-G, 22%. The haplotype C-G-C-T-C-A showed similar p-values for all the significant associations that we observed earlier for the two SNPs (see table 6.3).

Table 6.3

SSRI	N		rs2235040A	rs2032583C	C-G-C-T-C-A
P-gp substrates (i.e. paroxetine, venlafaxine, citalopram, fluvoxamine, sertraline)	424	adverse effects (0 vs 1+)	1.61 (p = 0.051)	1.73 (p = 0.028)	1.63 (0.052)
		adverse effects (0 vs 2+)	**2.32 (p = 0.002)**	**2.41 (p = 0.001)**	**2.35 (p = 0.002)**
		anticholinergic (0 vs 1+)	0.781 (p = 0.41)	0.782 (p = 0.41)	0.813 (p = 0.41)
		anticholinergic (0 vs 2+)	**1.92 (p = 0.011)**	**1.92 (p = 0.010)**	**1.92 (p = 0.011)**
		serotonergic (0 vs 1+)*	**1.85 (p = 0.005)***	**1.96 (p = 0.002)***	**1.93 (p = 0.005)***
		serotonergic (0 vs 2+)	1.88 (p = 0.16)	2.07 (p = 0.12)	2.08 (p = 0.12)
		histaminergic (0 vs 1+)	0.855 (p = 0.51)	0.85 (p = 0.50)	0.84 (p = 0.48)
		histaminergic (0 vs 2+)	1.88 (p = 0.089)	1.87(p = 0.094)	1.97 (p = 0.075)
		constipation	1.54 (p = 0.072)	1.55 (p = 0.66)	1.79 (p = 0.064)
		dry mouth	1.62 (0.046)	1.63 (p = 0.042)	1.69 (p = 0.052)
		sweating	1.34 (p = 0.27)	1.36 (p = 0.26)	1.33 (p = 0.31)
		restlessness	1.34 (p = 0.44)	1.35 (p = 0.43)	1.34 (p = 0.45)
		sleepless	**2.93 (p = 0.0003)**	**2.95 (p = 0.0003)**	**3.12 (p = 0.0003)**
		sleepiness	1.45(p = 0.12)	1.46 (p = 0.11)	1.53 (p = 0.083)
		weight gain	1.01 (p = 0.99)	1.00 (p = 0.99)	1.02 (p = 0.96)
		diarrhea	1.18 (p = 0.74)	1.21 (p = 0.71)	1.22 (p = 0.70)
		nausea	1.26 (p = 0.48)	1.41 (p = 0.29)	1.41 (p = 0.30)
		sexual	1.20 (p = 0.48)*	1.20 (p = 0.46)*	1.19 (p = 0.50)*
		twitching	1.48 (p = 0.23)	1.49 (p = 0.21)	1.51 (p = 0.21)
		dizziness	1.13 (p = 0.65)	1.23 (p = 0.44)	1.25 (p = 0.42
paroxetine	151	adverse effects (0 vs 1+)	1.45 (p = 0.36)	1.60 (p = 0.25)	1.45 (p = 0.36)
		adverse effects (0 vs 2+)	1.63 (p = 0.34)	1.76 (p = 0.23)	1.66 (p = 0.28)
		anticholinergic (0 vs 1+)	0.99 (p = 0.95)	0.98 (p = 0.96)	0.95 (p = 0.95)
		anticholinergic (0 vs 2+)	3.22(p = 0.034)	3.28 (p = 0.031)	3.27 (p = 0.033)
		serotonergic (0 vs 1+)	1.28 (p = 0.47)	1.36 (p = 0.38)	1.37 (p = 0.45)
		serotonergic (0 vs 2+)	n.a.	n.a.	n.a.
		histaminergic (0 vs 1+)	0.73 (p = 0.40)	0.76 (p = 0.46)	0.77 (p = 0.43)
		histaminergic (0 vs 2+)	n.a.	n.a.	n.a.

Single locus and haplotype results from multinomial and logistic regression analyses on SSRI-related side effects Significance was assessed using multinomial and logistic regression analysis adjusted for sex, age and statin use, and odds ratio's including p-values, are shown for minor allele carrier versus non-carrier. Bold values indicate significant results. n.a. = not analysed because of too low numbers; P-gp = P-glycoprotein; SSRI = specific serotonergic reuptake inhibitor. ***** significant sex effect (see results).

Discussion

Our results suggest that some *ABCB1* polymorphisms might predict adverse side effects related to the SSRIs that have affinity for P-glycoprotein. Of the six SNPs tested, two intronic SNPs (rs2235040A and rs2032583C) and the haplotype C-G-C-T-C-A were

significant predictors for the number of adverse side effects, and were in particular associated with serotonergic (especially sleeplessness) and anticholinergic effects.

To date, this is the first study demonstrating a relation between the pharmacokinetic gene *ABCB1* and adverse drug effects in SSRIs. The associations between the SNPs rs2235040 and rs2032583 and phenotype or clinical response, seem promising. In our study, the two SNPs were in near complete LD, thus representing the same information. Both SNPs have been associated with higher treatment response on antidepressants, that are transported by P-gp, in contrast to mirtazapine, which is not a substrate for P-gp.[11,12] In these studies, the T-carriers of rs2032583 were associated with poor treatment response, which may correspond with a higher level of adverse effects, as we found for the rs2032583 C-allele.

Neither our study nor previous ones,[11,12,35] found an effect of the SNPs 1236C>T, 2677G>T/A and 3435C>T. These three SNPs have been the focus of many pharmacokinetic and disease related studies, with contradictory results, since these are common SNPs in the coding region of the *ABCB1* gene.[36] However, genetic associations of these SNPs with clinical phenotypes have largely been inconsistent.[9,37] The 1236C-2677G-3435C haplotype is one of the most frequently observed in most populations.[37] In fact, the C-G-C haplotype has been connected to altered P-gp functionality in several studies, but *ABCB1* haplotype association studies have also been inconclusive so far.[9,36] In our study the haplotype containing 1236C-2677G-3435C links to the rs2032583C and rs2235040A allele and both these single SNPs and the total haplotype of SNPs were associated with adverse drug effects. This implies that the haplotype association that was observed previously might be caused by the association of the two single SNPs rs2032583 and rs2235040, which are in almost complete LD with each other. The intronic SNPs rs2032583 and rs2235040 are non-coding SNPs, but might be linked to another functional variant. However, in the absence of replication of the findings in this report, and in the light of the many controversies on *ABCB1* related gene effects so far, the conclusions need to be interpreted with care.

One of the limitations of this study is the fact we have not controlled for all co-medication. Many drugs have shown to posses some inhibitory or inductory properties *in vitro*, such as statins,[38] antipsychotics or antidepressants,[6] but their impact on P-gp function *in vivo* may be clinically irrelevant.[5,6] Therefore, we only excluded co-medication, for which clear evidence was available, with respect to P-gp inhibitory or inductory properties of a drug, e.g. antiretroviral or other immunosuppressive agents.[39] We controlled for statin use because this group was considered large enough to include in the analyses as a covariate. In none of the analyses, however, any interaction was seen. Interaction with other co-medication can however not be excluded. The gene

effect that we found for the SSRIs with affinity for P-gp may not be valid for each SSRI to the same degree. The selected SSRIs have shown to have comparable substrate properties[2,4-6] and moreover, substrate specific *in vivo* data on P-gp function is lacking.

We found an association between SNPs in *ABCB1* and serotonergic and anticholinergic activity. With respect to both receptor systems, there is no obvious relation (at least not for antidepressants) between serotonergic or anticholinergic activity and substrate affinity for P-gp or P-gp function. An explanation for the association with anticholinergic activity could be the overrepresentation of paroxetine (>35%) in the sample. Paroxetine exhibits higher anticholinergic activity than all other SSRIs.[40] The fact that we did not find an association in the subgroup of paroxetine users may be due to the sample size.

In conclusion, our results suggest that the number of adverse effects related to SSRIs, transported by P-glycoprotein, in particular serotonergic (especially sleeplessness) and anticholinergic effects, is associated with two intronic SNPs of the *ABCB1* gene (rs2235040 and rs2032583).

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Chapter 7

ABCB1 **gene variants influence tolerance to selective serotonin reuptake inhibitors in a large sample of Dutch cases with major depressive disorder**

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Abstract

Background: P-glycoprotein, an ATP-driven efflux in the blood-brain barrier, has a major impact on the disposition of antidepressant drugs in the brain. Gene variants of the encoding gene *ABCB1* have inconsistently been associated with both treatment response, side effects and have been suggested as vulnerability factor for depressive disorders.

Method: Data were from 1601 depressed patients of the Netherlands Study of Depression and Anxiety (NESDA). We explored a possible role of 6 *ABCB1* gene variants (1236T>C, 2677G>T/A, 3435T>C, rs2032583, rs2235040, rs2235015) as susceptibility factors in the course of MDD. Susceptibility indicators of MDD (young age of onset, recurrent episodes, familial aggregation) were tested for their association with the 6 polymorphisms and haplotypes.

Results: Of the possible susceptibility factors of MDD, 'young age of onset' was negatively associated with three SNPs rs2032583 ($p = 0.005$), rs2235040 $p = 0.005$), rs1045642 ($p = 0.0015$) and a haplotype ($p = 0.006$), indicating that the minor allele of these gene variants protected against early onset depression. Two haplotypes containing 1236T-2677T-3435T were significantly ($p = 0.0065$ and $p = 0.010$) associated with recurrent episodes.

Conclusion: Some *ABCB1* gene variants may indicate susceptibility for early onset and recurrent MDD.

Introduction

P-glycoprotein (P-gp) is an ATP-driven efflux pump, a product of the *ABCB1* gene, and is located at the luminal side of the brain capillaries. Among efflux transporters at the blood-brain barrier, P-gp plays a dominating role, since it transports hundreds of chemically diverse molecules. The physiological role of P-gp at the blood-brain barrier involves the protection of the brain from blood borne xenobiotics. In major depressive disorder (MDD), P-gp has a tentative role in the disposition and pharmacokinetics of antidepressant treatment, as many antidepressants have affinity for this pump.[1]
A protective role for P-gp in MDD may be less obvious, but its activity appears to decrease in specific brain regions with age, possibly indicating a role in neuroprotection.[2] Besides, genetic variation may influence the vulnerability for a disease. For example, *ABCB1* gene variants have recently been associated with higher risk of drug addiction.[3]
The evidence for the involvement of the *ABCB1* gene in MDD as a susceptibility factor is sparse. To date, two small studies have been published. In the first, a weak association (not corrected for multiple testing) between the 2677A allele and mood disorders has been described in a Japanese study of 62 subjects[4] and secondly, two single nucleotide polymorphisms (SNPs) (rs1202184 and rs1922242) were related to severity of MDD in a Taiwanese study.[5]

The relative risk for MDD is increased higher for first degree relatives of probands with MDD,[6] but is further increased in probands with an age at onset below 30 years or with recurrent episodes.[6-8] The subgroup with recurrent episodes and early onset is often considered as a narrow phenotype of MDD, with a higher relative risk for MDD, which may be 4-5 times higher than non recurrent, late onset MDD.[9] The higher relative risk in probands with a recurrent early onset MDD increases the power to map susceptibility genes.

With the availability of the NESDA data (Netherlands Study of Depression and Anxiety), a large study cohort with 1601 participants with a diagnosis of MDD, who had been selected for genome-wide genotyping, it is possible to examine SNP profiles within the ABCB1 gene that characterize susceptibility factors in MDD.

Methods

NESDA sample

The NESDA is a longitudinal, naturalistic cohort study examining the course and consequences of depressive and anxiety disorders in 2981 participants. The collection and genotyping of the NESDA sample has been described elsewhere.[10]

SNP selection and genotyping

A selection of six single nucleotide polymorphisms (SNPs) was made based on positive reports in the available literature.[4,5,11] This includes the three common coding SNPs 3435C>T (rs1045642), 2677G>T/A (rs2032582) and 1236T>C (rs1128503)[12] and three noncoding SNPs (rs2235015, rs2032583 and rs2235040) that were associated with treatment response on antidepressants.[13,14] The genotype data were obtained from the original genome-wide association study.[15] These data were subjected to a rigorous quality control criteria.[16] None of the six SNPs was genotyped, estimated genotype probabilities and allele dosages were obtained through imputation.[17] The quality of imputation was good (p=0.93) for 3435C>T and excellent (p=0.99-1.00) for the other SNPs. Details of the SNPs are presented in table 7.1.

Table 7.1

SNP	reference sequence	Position *	c/o	Coded allele frequency	HWE P	Imputation quality	role
3435	rs1045642	86976581	C/T	0.444	0.22	0.93	coding exon 27
2677	rs2032582	86998554	G/T	0.577	0.61	0.99	coding exon 22
1236	rs1128503	87017537	C/T	0.579	0.71	1.00	coding exon 13
	rs2235015	87037500	G/T	0.793	0.79	0.99	Intron 5
	rs2032583	86998497	C/T	0.134	0.82	1.00	intron 22
	rs2235040	87003686	A/G	0.134	0.82	0.99	intron boundary exon 21

Major characteristics of all single nucleotide polymorphisms

*Position from NCBI Genome build 36, release 22; c/o = coded allele versus other allele; HWE = Hardy-Weinberg equilibrium (p value)

Baseline and 2-year follow up data, collected between September 2004 and February 2007, were used. 1601 cases with a lifetime MDD were included. Of those, 786 had experienced one episode and 815 had had more than one episode. For all cases genotype information was available.

Three presumed indicators of susceptibility in MDD were examined in relation to the SNPs: 'age of onset' (defined as MDD starting before the age of 30)[6] and 'recurrence', 'familial risk' and 'treatment course'. The variable 'age of onset' was analyzed as

dichotomous variable ('MDD starting before 30 years' versus 'starting after 30 years' and as continuous variable. The variables 'recurrence' were (highly) skewed and therefore a multinomial variable was constructed. 'Recurrence' had four values (i.e. 0 (= first episode), 1, 2 and 3 (three or more recurrences). This classification was chosen, because three or more depressive episodes is often regarded as chronic depression, resulting in other treatment algorithms.[18] The variable 'familial risk' indicated the number of first degree family members and had three values (0, 1 (1 or 2 family members) and 2 (≥ 3 family members). We did not construct a multinomial variable 'age of onset', because this variable was normally distributed and besides, differentiation between 'starting before 30 years' and 'starting after 30 years' seemed most discriminant.[6] Age and sex were used as covariate.

Statistical analysis

For the continuous variable 'age of onset' linear regression was used, and the other variables (dichotomous variable 'age of onset', 'familial risk' and 'recurrences' were analyzed with multinomial regression analysis with SNPs as independent variables. Frequencies of the haplotypes containing the all 6 SNPs were estimated using the following randomization procedure. For each individual and each SNP a genotype was assigned randomly five times from its imputed genotype probability distribution. Next for each randomized set the software package PHASE was used to estimate haplotype probabilities for each individual.[19] Finally the haplotype probabilities were aggregated and dosages of the eight possible haplotypes were calculated (i.e. yielding eight variables). Haplotype analysis was performed for the three most common haplotypes with a frequency of >10% using linear or multinomial regression analysis, similar to the single SNP analyses described earlier. We also determined the haplotype frequencies of all 6 SNPs[20] and used these frequencies to estimate linkage disequilibrium (LD) of each pair of SNPs.

Given the complete LD for one SNP pair (see results), an adapted approach for multiple testing was applied,[21] and the significance threshold was set at 0.0127. Statistical analyses were conducted using SPSS software version 16.0 (SPSS Inc., Chicago, Illinois, USA) and Microsoft Excel® 2003 (Microsoft corporation, Redmond, Washington, USA).

Results

At baseline, 786 subjects had a first depressive episode and 815 had undergone one or more recurrences. The sample consisted of 1099 females and 502 males. Mean age of onset was 27.5 ± 12.3, the average age at the time of the interview was 42.0 ± 12.5. Of the 1601, 366 had a negative (first degree) family history for MDD, 868 had 1 or 2 affected family members, and 367 had more than 2 family members with MDD.

Susceptibility factors of MDD

'Age of onset' was significantly associated with rs2235040A (p = 0.005, beta = -0.070) and rs2032583C (p = 0.007, beta = -0.067), indicating that these carriers had a higher on onset of MDD at younger age. The subjects with MDD, who were carrier of 3435C or rs2235040A had a higher change on onset of MDD starting before 30 years (n = 889). The Odds ratio (OR) for 3435C carriers was 1.27 (p =0.0015) and for rs2235040A OR: 1.31 (p = 0.01). In both analyses male sex was significantly associated with lower change on early onset (OR = 0.60; p = 0.00004). For none of the SNPs an association was found with either 'recurrences' or 'family risk'. Results are presented in table 7.2.

Haplotype analysis

The r^2 values between the different SNPs were high for 2677-1236 (r^2 = 0.88), suggesting strong LD. rs2235040 and rs2032583 (r^2 = 0.98) demonstrated nearly complete dependency. The other r^2 values ranged from 0.10 to 0.56. Five haplotypes containing the three most common coding SNPs (1236C>T-2677G>T/A-3435C>T-rs2235015-rs2032583-rs2235040) and capturing a large proportion of the observed haplotypes[12] had an observed frequency of >5 % and were examined in relation to the examined variables. The frequencies of the different haplotypes were: C-G-C-G-T-G (22%), T-T-T-G-T-G (39%), T-G-C-G-T-G (14%), C-G-C-T-T-G (6.4%) and C-G-C-T-C-A (13%), which (pertaining to the 1236-2677-3435 haplotype) were comparable to the frequencies reported in the literature.[12] The C-G-C-G-T-G and C-G-C-T-T-G haplotypes appeared significantly associated with 'recurrence' (p = 0.010 and p = 0.0065).

No association was found with 2 or more recurrences, which was not related to power, the numbers of subjects with 1,2 of 3 and more episodes were comparable (1 recurrence: 257; 2 recurrences: 227 and ≥3 recurrences: 328 subjects). Age had a negligible effect (OR = 1.02; p = 0.002) and sex had no effect. The haplotype C-G-C-T-C-A was significantly (negatively) associated with continuous age of onset of depression (beta = -0.068; p = 0.006), as well as with 'MDD starting before 30 years' (OR = 1.31; p = 0.012), with lower risk for male gender (OR = 0.60; p = 0.00004). The haplotypes T-T-T-G-T-G and C-G-C-G-T-G showed a non significant association with

intermediate family risk (p = 0.027) and low familiy risk (p = 0.049), respectively (see table 7.2).

Discussion

In the present study we found significant associations between several polymorphisms of the *ABCB1* gene and onset of MDD before 30 years (rs2235040A: OR= 1.3, p = 0.01; 3435C: OR = 1.3, p = 0.0015) or onset of MDD at younger age (rs2032583C; beta = -0.07, p = 0.007; rs2235040A: beta = -0.067, p = 0.007). For the haplotype C-G-C-T-C-A comparable associations with age of onset were found. Furthermore, the haplotypes C-G-C-T-T-G (OR = 1.7, p = 0.0065) and C-G-C-G-T-G (OR = 0.71; p = 0.01) were associated with recurrent MDD. These results suggest that several polymorphisms and a haplotype of the *ABCB1* gene may either predict onset of MDD at younger age (or before 30 years) and recurrence of MDD.

The differences we found in *ABCB1* gene variation between the group with first episode MDD and those with one recurrence, as well as between those with an early onset may indicate a relation between the *ABCB1* gene and an unfavourable course of the disorder or severity. It is unknown whether the studied *ABCB1* polymorphisms are also connected to increasing the risk for MDD, since we did not compare between MDD phenotypes and control cases. In respect to recurrent MDD it is unclear why no gene effect is seen for the groups with two or more recurrences, but is conceivable that other (gene) effects interfere with a possible adverse effect of *ABCB1* gene variants.

Higher remission percentages for rs2235040A and 2232583C carriers were found for MDD patients treated with an antidepressant with P-gp substrate properties.[13,14] In a recent study (de Klerk *et al.*, unpublished observations) we found an association between these SNPs and SSRI-related adverse effects. No effect on treatment response for these genes was found for duloxetine,[22] which is probably not a P-gp substrate. The results for SNP 3435C>T as predictor of treatment response have been discordant, only one study describes a higher remission rate in treatment with either paroxetine, venlafaxine, or (es)citalopram for C-carriers (haplotype including 3435CC).[5,11,13,14,23,24]
A possible link between early onset of depression, higher level of adverse drug reactions on SSRIs with P-gp affinity, and higher remission rates on SSRIs could imply that the SNPs rs2235040 and rs2032583 are associated with decreased function of P-gp at the blood-brain barrier. A decreased P-gp function may lead to higher net influx of an antidepressant, thus attributing to higher remission rate, and also to higher (central)

adverse drug reactions. Besides, there are theories suggesting a relation between vulnerability for neuropsychiatric disorders and a defective blood-brain barrier.

Table 7.2

		rs2235040	rs2032583	rs1045642	rs2032582	TTTG TG 38.9%	TGCG TG 13.8%	CGCG TG 22.2%	CGCT CA 12.9%
recurrent episodes	0-1	0.90; 0.49	0.91; 0.54	0.86; 0.15	0.92; 0.40	1.02; 0.83	**1.7; 0.0065**	**0.71; 0.010**	0.93; 0.63
	0-2	1.00; 0.99	0.99; 0.95	0.97; 0.76	1.11; 0.33	1.09; 0.50	1.11; 0.66	1.00; 0.65	0.99; 0.95
	0-3	0.94; 0.67	0.95; 0.73	0.96; 0.65	1.01; 0.89	0.90; 0.42	1.44; 0.054	0.86; 0.11	0.96; 0.77
age of onset	*<30	**1.32; 0.010**	1.29; 0.015	**1.27; 0.0015**	0.86; 0.085	1.22; 0.023	0.84; 0.25	1.17; 0.078	**1.31; 0.012**
	**age	**-0.070; 0.005**	**-0.067; 0.007**	-0.062; 0.014	-.0042; 0.092	0.48; 0.057	0.035; 0.17	-0.020; 0.42	**-.068; 0.006**
Family risk	0-1	1.00; 0.99	0.91; 0.99	1.12; 0.16	1.13; 0.14	0.87; 0.087	0.92; 0.62	1.22; 0.049	0.98; 0.89
	0-2	1.03; 0.86	1.01; 0.94	1.08; 0.46	1.27; 0.027	0.78; 0.027	1.04; 0.28	1.16; 0.28	1.03; 0.87
	0-3	0.89; 0.48	0.89; 0.48	0.97; 0.97	0.98; 0.86	0.74; 0.96	1.17; 0.27	1.17; 0.27	0.86; 0.37
MDD with early onset and 2 episodes	0-1			0.98; 0.87			**1.8; 0.012**	0.80; 0.15	
	0-2			0.72; 0.033			1.6; 0.072	**0.59;0.012**	

Single locus and haplotype results for regression analyses on susceptibility factors in MDD Significance was assessed using multinomial regression analysis, and odds ratio's including p-values, are shown for minor allele carrier versus non-carrier. Bold values indicate significant results. *logistic regression (MDD starting before 30 years versus starting after 30 years) ** linear regression analysis was used for the continuous variable 'age of onset'

Furthermore, the suggested gene effect are limited, because the odds ratios are rather small. Due to the small effects, these polymorphisms are likely not useful as single indicator in clinical practice. Nevertheless, the uncovering of associations in *ABCB1*

and other genes, each conferring a small effect on the MDD phenotype may help to point out susceptibility factors in the disorder.
In conclusion, our results suggest that the polymorphisms rs2235040, 2232583 and 3435CC and the haplotypes 1236-2677-3435 T-T-T and T-T-C may be related to altered susceptibility for MDD.

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Chapter 8

Chronic stress and antidepressant treatment have opposite effects on P-glycoprotein at the blood-brain barrier

an experimental PET study in rats

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Abstract

Background: The multidrug efflux transporter P-glycoprotein (P-gp) is expressed in high concentrations at the blood-brain barrier (BBB) and has a major function in the transport of drugs. In a recent PET-study evidence was found for an increased function of P-gp at the BBB in medicated patients suffering from major depressive disorder. We used small-animal PET and [^{11}C]-verapamil to study P-gp function at the BBB of rats, either being administered venlafaxine, an antidepressant, or subjected to chronic stress, a factor contributing to the development of depression.

Methods: In a first experiment, male Wistar rats underwent a 3–week footshock procedure as a model of human depression. In a second experiment, rats were chronically treated with the antidepressant venlafaxine (25 mg/kg/d via an implanted osmotic minipump). In both experiments. Thereafter, a [^{11}C]-verapamil PET scan was performed. Results: In the chronically stressed rats, the distribution volume (V_T) of [^{11}C]-verapamil was significantly increased, whereas treatment with venlafaxine had the opposite effect and caused a significant reduction in V_T. The changes in V_T could not be attributed to the influx rate constant (K_1).

Conclusion: Our data suggest that P-gp function at the BBB is inhibited by chronic stress and increased by chronic administration of venlafaxine.

Introduction

In normal circumstances the blood-brain barrier (BBB) contributes to brain homeostasis by protecting the brain from potentially harmful endogenous and exogenous substances.[1] This restricted entrance to the brain is mainly established by active efflux mechanisms at the BBB.[2] One of the major efflux transporters is P-glycoprotein (P-gp), acting as a gate keeper protein for toxic substances at the luminal side of the brain capillary.[3] Due to its localization, potency and broad multispecificity P-gp is a key determinant of drug entry to the brain.[4]
P-gp function can be modulated by a wide variety of endogenous and environmental stimuli,[5] including proinflammatory cytokines.[6,7] In vitro, the P-gp function at the BBB is downregulated after short term exposure to inflammation whereas its function is upregulated following more prolonged exposure.[8-10]

Depressive disorders have been linked to neuroinflammatory events, wherein proinflammatory cytokines, acute phase proteins and possible endotoxic substances easily cross the BBB due to an increased permeability.[11,12] Other indications for a loss of integrity of the BBB in depressive disorders come from studies of the cerebrospinal fluid (CSF) in depressed patients.[13-15]

In a PET study with [^{11}C]-verapamil, recently performed by our group,[16] we found an increased function of P-gp at the BBB in a group of medicated patients with a major depressive disorder (MDD). The interpretation of this finding was complicated by the heterogeneity in the patient group and the use of antidepressants and other psychotropic drugs. There are in fact two explanations that could account for the change in P-gp function we found. First, the increase in P-gp function might be a result of the depressive disorder itself or a result of some sort of neuronal or physiological correlate of depression, e.g., chronic neuroinflammation or stress. Second, and opposite to the first explanation, the increase in P-gp function might be a result of antidepressant treatment.
Most antidepressants are substrates of the P-gp pump and generally they have a weak inhibitory effect.[17,18] However, so far this effect is only found in vitro with concentrations that are far above therapeutically relevant levels.[19-21]
In the present study we aimed to differentiate between the two explanations for the increase in P-gp function in depressed patients. With laboratory rats as a model species, we used an experimental approach in a strictly controlled setting to examine changes in P-gp function in response to: 1) chronic antidepressant treatment, and 2) chronic, uncontrollable stress, as a potential contributing factor to the development of depression. We hypothesised that chronic stress would lead to inhibition of P-gp function and that chronic antidepressant treatment at a therapeutic level would lead to

increased P-gp function. [^{11}C]-verapamil positron emission tomography (PET) has been found to be a suitable methodology for the in vivo assessment of P-gp function.[22,23] [^{11}C]-verapamil is a well characterized PET ligand for evaluating P-gp function at the BBB; it is a poor substrate for other transporters such as Multi Resistance Protein (MRP1).[23-25]

To assess these hypothesized changes in P-gp function, PET brain imaging with [^{11}C]-verapamil as radiotracer was performed in two study groups, i.e. a chronic stress model and a continuously administered antidepressant model, and two control groups of male Wistar rats. The distribution volume of [^{11}C]-verapamil was used as a measure of total P-gp function.

Methods

Subjects

48 Male Wistar rats (Harlan, Groningen, the Netherlands) weighing 275-300 g at the time of arrival, were individually housed in cages with food and water ad libitum. The room was maintained at a temperature of 21 ± 1 °C and on a 12:12 dark/light cycle. Housing conditions were the same for all rats. Animal experiments were conducted according to the European Council Directive of November 24, 1986 (86/609/ECC) and were approved by the Committee on the Ethics of Animal Experiments of Groningen University.

Induction of stress

The footshock stress procedure was done using a footshock box containing an animal space placed on a grid floor connected to a shock generator and scrambler. This stress model was adapted from Trentani et al.[26] Stress group rats (n=16) were subjected to a daily session of footshock stress for 21 consecutive days. During the session in the footshock box rats received 5 uncontrollable and inescapable footshocks (0.8 mA in intensity and 8 s in duration). All footshock sessions took place during the light phase. In order to increase unpredictability and minimize habituation, both duration of footshock sessions and intervals between shocks within a session varied randomly (session duration: 15-80 min; shock interval: 1-15 min). Control rats stayed undisturbed in their home cages throughout the experiment. In the beginning of the light phase, before the start of daily footshock session, all rats were weighed and the difference in body weight gain between the groups was used as a read-out of stress effect on animals' physiology.

Administration of venlafaxine

To study the effects of chronic antidepressant treatment on P-gp function, rats were subjected to a 3-week treatment on venlafaxine. After 14 days of acclimatization a group of 8 rats were anesthetized with Isoflurane (2-2.5%). Osmotic minipumps (Alzet Model 2004, purchased from Charles River) containing 250 mg venlafaxine (kindly provided by Dr. F.J. Bosker) dissolved in 2 ml saline (0.9 % NaCl) were subcutaneously implanted. This pump model continuously releases its content at approximately 2.5 µl/hr for 4 weeks. Assuming that the average body weight of the rat is around 360 g during the procedure, the mean administered dose of venlafaxine is 25 mg/kg/day. We estimated that this dose would approach the human therapeutical serum level.[27,28] Before implantation, all pumps had been placed in a sterile 37°C saline bath for 2 days. The control group (8 rats) underwent the same procedure, the osmotic minipump containing saline without an active drug. The active drug or the vehicle was administered for 21 days.

Radiochemistry

Racemic [^{11}C]-verapamil was produced as previously described.[29] The injected radioactivity of [^{11}C]-verapamil varied between 50-100 MBq with a specific activity of at least 40 GBq/nmol.

PET procedure

On the day of the PET scan the rats were transported in their home cage to the PET facility. The transport was at least 4 hours prior to the scan procedure, so that the animals could habituate to their new environment. Before the PET procedure, the rats were anesthetized with 2-2.5% isoflurane, whereupon a canula for arterial blood sampling was inserted into the right femoral artery. All scans were performed with a Focus 220 microPET camera (Siemens/Concorde Microsystems, Knoxville, USA). After injection of [^{11}C]-verapamil in the penile vein serial dynamic PET scanning was performed at escalating time frames and serial arterial blood sampling for [^{11}C]-verapamil took place during the scan in order to define the input function. Of each animal 14 samples were manually drawn at t = 15, 30, 45, 60, 75, 90, 120, 180, 300, 450, 600, 900, 1800 and 3600 s. The blood volume of each sample taken, around 0.1 ml, was partially replaced by saline/heparine, to prevent clotting of the canula.

These samples were further processed to measure the radioactivity in plasma. In this way the contribution of the injected activity to the PET-signal could be calculated. Metabolite analysis could not be realized since this would lead to hypovolemia in the rat during scanning. In each PET scan session two animals were scanned, the first rat starting at t=0, the second starting at t = 960 s (one control and one experimental rat, in random order to prevent a scan order effect). The injected dose of [^{11}C]-verapamil at the moment of injection was similar for both animals to ensure similar counting statistics. All data were corrected for physical decay to the moment of injection.

Metabolite correction

Determination of metabolites in the rats used in the study was not feasible, because of the risk of hypovolemia during scanning. Instead, we used metabolite data performed in three control rats (provided by Dr. G. Luurtsema). Mean percentages of metabolites and parent compound at four time points were used to construct a standard metabolite curve, using SigmaPlot (Systat Software Inc., San Jose, USA) (option 'exponential rise to max'). This regression curve was used as standard metabolite correction for the plasma input function in the modelling. Subsequently we manipulated this curve and constructed a regression curve, reflecting a hypothetical situation of a 'slow' metabolism, corresponding to a 5-10% diminution of metabolites formed after 60 minutes of scanning. By using this curve as input in the venlafaxine arm of the study (the normal metabolite curve as input for the control group), we could make an estimation of the V_T difference across the two groups in case metabolism would be substantially slowed down through the administration of venlafaxine.

List mode data were reframed into a dynamic sequence of 4 x 60 s, 3 x 120 s, 4 x 300 s, 3 x 600 s frames. Images were reconstructed per time frame employing an interactive reconstruction algorithm (OSEM2D). The final datasets consisted of 95 slices with a slice thickness of 0.8 mm, and an in-plane image matrix of 128 x 128 pixels of size 1 x 1 mm^2. Data sets were fully corrected for random coincidences, scatter and attenuation. A separate transmission scan was acquired for attenuation correction. This scan was performed right before the emission scan. A study-specific template was constructed onto which the realigned images were spatially normalized.

Data analysis

After conversion to an ECAT7 image file format the microPET images were analyzed with a ROI-based approach (regions of interest). A whole brain ROI was manually drawn using Clinical Applications Programming Package software (CAPP5; CTI/Siemens PET Systems, Knoxville, TN). A graphical analysis according to Logan for quantification of the dynamic PET data was done with plasma data as input.[30] The Logan plot was started at 5 minutes and the parameter for cerebral blood volume was fixed at 0.0. With this method the distribution volume (V_T) was estimated. Because the slope (i.e.V_T effect) obtained in the graphical approach may be biased in the presence of noisy data,[31] we verified the V_T in a kinetic analysis (i.e. single tissue compartment model). The influx rate constant (K_1) and efflux k_2 were derived from this model and on all parameters (i.e. V_T, K_1 and k_2) the group means (experimental group vs. control group) were compared with each other, using t-tests. Analysis of variance was done to test for the influence of K_1, k_2 and body weight gain (or venlafaxine concentration) as a predictor of V_T in the stress group (resp. venlafaxine group).

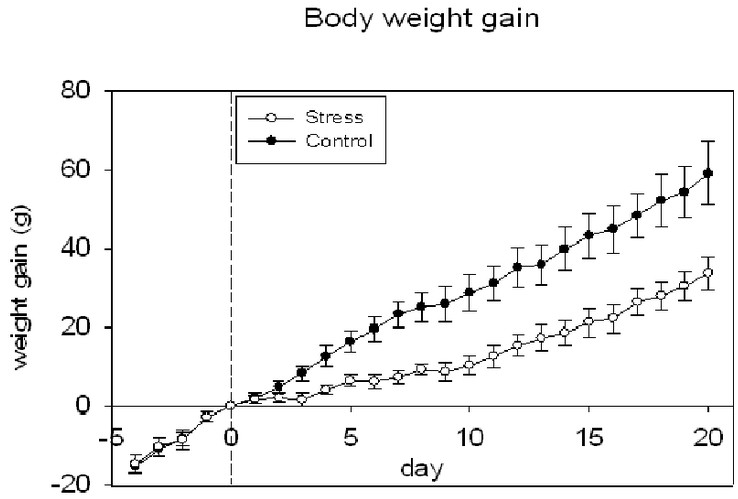
Results

Effects of chronic stress

The stress procedure was started with 16 rats (35 days, 448.1 g ± 26.3) and 16 rats (416.3 ± 32.1) were used as control. Statistics were performed using analysis of variance (repeated measures) for the body weight gain during the stress procedure. The chronically stressed rats gained less weight than the control rats; 30.1 ± 14.4 (stress) vs. 60.1 ± 25.2 g (controls); $p = 0.004$ (t-test), (see figure 8.1). Also, a significant effect of time was seen ($F=93.46$; $p<0.001$).

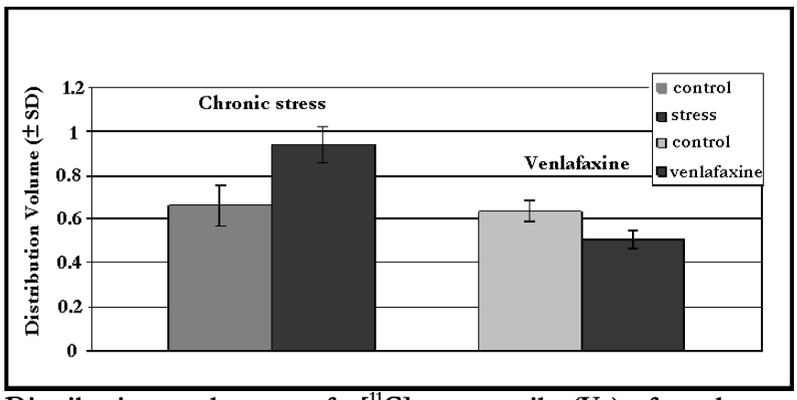
11 of the controls and 10 stressed rats could be used for the final analysis. The Logan gave a good fit in all cases. A significant increase in V_T was found in the stress group (V_T (stress): 1.40 ± 0.54 versus V_T (controls): 1.00 ± 0.30; $t= 2.14$; $p = 0.047$) (see figure 8.2). K_1 and k_2 did not differ across the two groups; K_1 was 0.28 ± 0.24 (controls) vs. 0.13 ± 0.06 (stress); $p = 0.11$ and k_2 was 0.21 ± 0.14 (controls) vs. 0.16 ± 0.11 (stress); $p = 0.42$. A comparison was made between the V_T obtained from the Logan method with the V_T from a kinetic analysis (one tissue model). The Pearson's correlation coefficient was 0.990 and a nearly equal level of significance was reached when comparing the two groups in a t-test ($p = 0.051$). In an analysis of variance, body weight or weight gain had no significant predictive value for V_T.

Figure 8.1



Effect of stress on body weight gain. Chronically stressed rats (n=12) gained less weight than control rats (n=12). The animals were weighed every day starting 4 days before the first stress procedure (day 0, dotted line). Depicted are means ± SEM.

Figure 8.2



Distribution volumes of [^{11}C]-verapamil (V_T) for the stress and venlafaxine experiments, showing increased V_T in chronically stressed rats, and a significant decrease of V_T in rats receiving venlafaxine.

Venlafaxine administration

Eight rats of both groups had an osmotic minipump implanted either venlafaxine or saline. In one scanned pair a failure in computer software made the results questionable. The production of [^{11}C]-verapamil failed twice, leaving 5 rats per groups for analysis, of which 3 'venlafaxine rats' were scanned first. The average plasma concentration of venlafaxine was 77.6 ± 19.6 µg/l. The Logan fit was good in all cases. The V_T in the venlafaxine group (0.80 ± 0.14) was significantly lower than the V_T in the control group (1.00 ± 0.13), (t-test, p = 0.047) which was comparable to the control group of the stress experiment (see figure 8.2). The V_T in Logan showed a good correlation with V_T measured in a 'one tissue model' (Pearson's = 0.994, p = 0.000-). In the kinetic model the V_T in the venlafaxine group showed a comparable significant decrease in the t-test compared to the controls (t=-2.55; p = 0.039). K_1 and k_2 were not significantly lower in the venlafaxine group, although K_1 showed a trend towards significance: K_1 (controls) 0.14 ± 0.08 vs. K_1 (venlafaxine) 0.057 ± 0.01; p = 0.085; k_2 (controls) 0.17 ± 0.13 vs. k_2 (venlafaxine) 0.07 ± 0.02; p = 0.15. The measured venlafaxine concentration (after scanning) had no predictive value for V_T in an analysis of variance.

Subsequently we tested the differences in VT between the groups. With the venlafaxine arm with a signicantly slower metabolism (i.e. 5-10% decrease in metabolites formed after 60 minutes). The V_T in the venlafaxine group was still significantly lower (0.76 ± 0.13) than the control group (1.00 ± 0.13) which was significant in the Logan analysis (p = 0.036), but not in the kinetic analysis (t-test, p = 0.15).

Discussion

The present study shows that chronic stress in rats significantly increases the V_T of [^{11}C]-verapamil in the brain, whereas a sustained 3-week treatment with the antidepressant venlafaxine in a 'therapeutical' concentration decreases the V_T of the tracer. The increased V_T in chronic stress can be interpreted as a decrease in P-gp function at the BBB, since no differences in influx (K_1) were seen between the groups. For the venlafaxine treated rats the decreased V_T may either be explained by an increase in P-gp activity and/or an increased influx, since K_1 showed a nearly significant decrease in the treatment group.

The chronic footshock stress model used in this study has been previously shown to induce HPA axis hyperactivity as well as impairments in the mechanisms governing neuroplasticity in the adult brain.[26,32,33] A highly significant difference in body weight gain between stress and control rats observed in our study has been described previously and demonstrates a strong stress effect on animals' physiology. Our results suggest that chronic stress leads to reduced P-gp function, which in the long run might leave the brain more vulnerable to the influx of potentially harmful substances. This reduction in BBB function might be one of the pathways via which stress contributes to central nervous system malfunction and disease. Our results may thus give new insight in the pathophysiology of depression

Increased P-gp function in response to venlafaxine administration should result in enhanced neuroprotection. However, increased pump function also implies reduced access of venlafaxine to the sites of action within the central nervous system. Chronic administration of venlafaxine may be related with a decreased pharmacological effect in the face of therapeutic doses, and consequently contribute to treatment resistance. The effects of venlafaxine treatment and chronic stress on P-gp are in the opposite direction. It is a plausible assumption that venlafaxine reduces the effect that chronic stress has on P-gp. The results suggest that our previous [^{11}C]-verapamil PET-study showing an increased P-gp function in medicated patients with chronic depression may thus be ascribed to the use of antidepressants.[34]

Several authors have demonstrated that antidepressants, can exert an inhibitory effect on P-gp function, although this has only been shown in vitro, at concentrations in the range above therapeutic conditions.[20,21] Venlafaxine has been shown to have a weak effect op P-gp, both inhibitory[21] and inductive.[35] The average serum level of venlafaxine in the rat was 69 μg/l, which is considered to be within the therapeutic range in humans.[28]

Moreover, chronic stress dysregulates the HPA-axis and leads to an increased glucocorticoid serum level.[36] Cortisol is a substrate of P-gp and in case of inhibition of P-gp its access to the brain is increased.[37,38]
Elevated levels of intracerebral cortisol are associated with compromised brain function and with neurochemical and anatomical changes in different brain areas.[39,40] An increased cortisol level also leads to an increased activation of brain glucocorticoid receptor function and an increased negative feedback on the HPA axis.[38] Although this feedback mechanism is altered in depression, antidepressants decrease HPA-activity in depression.[41] Cortisol may be regulated by P-gp through the inhibiting effect of antidepressants on P-gp. However, cortisol entry to the brain is probably not regulated by P-gp only, as Mason et al. found in mice.[42]

Metabolite measurement in the same animals was not feasible. In the absence of individually metabolite-corrected plasma input data our data are not entirely reliable, because a pharmacokinetic effect on verapamil metabolism through e.g. inhibition of P450 enzymes or an alteration of plasma free fraction cannot be excluded. However, significant changes in metabolism due to venlafaxine administration are not reasonable on the basis of the available literature. An effect of venlafaxine on the free fraction of [^{11}C]-verapamil is theoretically conceivable, although in verapamil metabolism the fraction is low (around 10%). Usually, the free fraction is not accounted for in modelling, because it can be assumed that there is an equilibrium is between the compartments. To date, no reliable method for determination of the free fraction of [^{11}C]-verapamil has been described.

Venlafaxine is a substrate of cyp2D6 and has a weak inhibitory effect on 2D6, whereas this iso-enzyme does not play an appreciable role in verapamil metabolism, which is mainly dependent on cyp3A4 metabolism.[43,44] Venlafaxine has no effect on 3A4 metabolism.[43,45,46]

Thus, although we had no reasons to suspect significant changes in [^{11}C]-verapamil metabolism through venlafaxine, we have compared the VT between the group in a hypothetical situation of a significant slower metabolism in the venlafaxine group
We still found a decrease of V_T in the venlafaxine group, which was significant in the Logan analysis ($p = 0.036$), although this could not be confirmed in the kinetic analysis ($p = 0.15$).

In conclusion, our results suggest that chronic stress in rats leads to a loss of integrity of the blood-brain barrier due to a decreased function of P-glycoprotein. The implication of this might be an increased brain vulnerability and increased sensitivity to central nervous system malfunction and disease. This finding supports the hypothesis that

reduced BBB-function might contribute to the pathophysiology of stress related disorders such as depression. Furthermore, our results suggest that antidepressants may in part exert their therapeutic effect by normalization of P-gp activity and BBB-function. Further studies using other antidepressants form different pharmacological classes are warranted to confirm our findings.

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Chapter 9

Distribution and function of P-glycoprotein at the blood-brain barrier in three animal models

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In preparation

Introduction

The blood-brain barrier (BBB) plays an important role in maintaining the internal milieu of the brain. One important function of the BBB is to restrict the entry of xenobiotics. P-glycoprotein (P-gp) is a multispecific efflux pump, highly expressed at the BBB and with the ability to extrude a wide array of unrelated compounds. In particular, lipophilic molecules, such as antineoplastic agents, immunosuppressants, cardiac glycosides, but also antidepressants and antipsychotics are substrates for P-gp.[1]

In humans, P-gp is encoded by a single gene, ABCB1, formerly denoted as MDR1. It belongs to the superfamily of ATP binding Cassette (ABC) transporters. Rats have two P-gp genes at the BBB, called *mdr1a* (or *mdr3* or *ABCB1a*) and *mdr1b (*or *ABCB1b)*. Together, these two genes have a comparable tissue distribution as human ABCB1. *ABCB1a* knockout mice have no detectable P-glycoprotein in the brain, indicating that this is the only gene responsible for drug efflux at the BBB.[2] ABCB1a encodes two isoforms of P-gp, both present in the BBB. The 140kDa protein appears to be the main isoform, it seems to be the immature form, which is not finally processed within the cell (also called called mdr3) (Potschka, personal communication). The 174 kDa protein is the functional mature form, which is integrated into the membrane, formerly named mdr1a.[3]

There is still some controversy regarding the exact localization of P-gp in the brain. The majority of studies have shown that its predominant location is at the luminal side of the endothelial cell,[4] but P-gp might also be involved in the regulation of drug transport at the subcellular level.[5]

P-gp is present in all BBB-protected regions, which excludes the choroid plexus, selected circumventricular organs and the posterior pituitary,[6] which have leaky capillaries. Under normal circumstances, P-gp is probably equally distributed throughout the brain, although the evidence so far is limited. In two recent PET studies in healthy controls, using R-[^{11}C]-verapamil as probe for P-gp function, regional differences in tracer uptake were neither seen at baseline nor following P-gp inhibition.[7,8]

Assessment of regional differences in P-gp expression and activity is of great interest, because a regional change in activity might contribute to the pathology of various neuropsychiatric disorders, but it could also be an indicator of local(ized) pathology. In PET studies indications were found for regional changes in P-glycoprotein activity with both psychiatric and neurological disorders.[9-11] Although not confirmed in PET studies, epilepsy and Alzheimer dementia might also be characterized by regional alterations in P-gp expression or function. Regional decline of P-gp function may also be related to ageing.[12,13]

It is now well established that many cortical and subcortical structures are involved in the psychopathology of major depressive disorder (MDD).[14,15] Brain activation studies

show hypoactivity in the frontal and temporal cortex as well as in the insula and cerebellum with patients suffering from MDD, whereas activity in these regions is increased during antidepressant treatment. The area and level of brain activation may depend on the drug of choice.[16,17] Cortical and subcortical areas have also been implicated in schizophrenia, in particular, the dorsolateral prefrontal cortex[18] and the basal ganglia.[19]

In this study we assessed the P-gp expression and the cerebral distribution patterns in three different models in male Wistar rats: 1) a depression model (chronic stress paradigm)[20] 2) a schizophrenia model (neuroinflammation paradigm)[21] and 3) an antidepressant treatment model[20] (3 week chronic administration of venlafaxine). Each condition might reflect physiological processes or localized pathology. Both P-gp function *in vivo* (using [^{11}C]-verapamil PET) and P-gp expression (using western blotting and immunohistochemistry) were used for comparisons. We have used the PET data on chronic stress and on chronic administration of venlafaxine and the rat brain tissue from a previously published animal PET study.[20]

Materials and methods

Animals

All experiments were performed on male Wistar rats (Harlan, Groningen, the Netherlands) weighing 275-300 g at the time of arrival. Animals were individually housed in cages with food and water ad libitum. The room was maintained at a temperature of 21 ± 1 °C and on a 12:12 dark/light cycle. Housing conditions were the same for all rats. Animal experiments were conducted according to the European Council Directive of November 24, 1986 (86/609/ECC) and were approved by the Committee on the Ethics of Animal Experiments of Groningen University.

Chronic stress procedure

This has been described in our previous publication.[20] Briefly, a group of rats (n=16) was subjected to a daily session of foot shock stress for 21 consecutive days. During the session in the foot shock box rats received 5 uncontrollable and inescapable foot shocks. Control rats (n=16) stayed undisturbed in their home cages throughout the experiment.

Chronic administration of venlafaxine

This has also been described in the previous study.[20] In short, osmotic mini-pumps were subcutaneously implanted in rats (n=16). These pumps continuously released either venlafaxine (25 mg/kg/day) (n=8) or saline (n=8) in the control group. The active drug or the vehicle was administered for 21 days.

Neuroinflammation model (Herpes encephalitis)

On the first day a group of 8 rats was intranasally inoculated with $1x10^7$ PFU of Herpes Simplex Virus-1, leading to an clinical encephalitis. A control group (n= 8) was inoculated with PBS. On day 6 both groups underwent a PET scan.

PET procedure and data analysis

The PET procedure has been described elsewhere.[20] In brief, before the scan the rats were habituated to the PET facility. After being anaesthesized the tracer [^{11}C]-verapamil was injected into the penile vein. 14 arterial samples and metabolite data were used as input function. A study-specific template was constructed onto which the realigned images were spatially normalized.

The reconstructed images were analyzed with a ROI-based approach (region of interest) with the use of a microPET program expanded with addition of Siemens Inveon Research Workplace (Siemens AG, Erlangen, Germany). This system is a multimodality image review, fusion, supporting CT and PET formats and it allows quantitative analysis of the data, using semi-automated region of interest techniques. 16 ROIs were predefined on a template. A graphical analysis according to Logan for quantification of the dynamic PET data was done with plasma data as input. The Logan plot was started at 5 minutes and the parameter for cerebral blood volume was fixed at 0.03. With this method the distribution volume (V_T) was estimated. Because the slope (i.e.V_T effect) obtained in the graphical approach may be biased in the presence of noisy data, we verified the V_T in a kinetic analysis (i.e. single tissue compartment model). The influx rate constant (K_1) and efflux k_2 were derived from this model and on all parameters (i.e. V_T, K_1 and k_2) the group means (experimental group vs. control group) were compared with each other, using t-tests.

Brain collection, western blotting and immunohistochemistry

At the end of the PET experiments, rats were still anaesthesized. Brains were dissected and divided into two hemispheres. The left hemisphere was dissected in three parts, which were hippoacampus, prefrontal cortex and parietal cortex, and was snap frozen in liquid nitrogen and conserved at -80˚C. The right hemisphere was fixed in 4% paraformaldehyde solution and was also cryopreserved.

For western blotting (WB) analysis whole brain tissue was homogenized, and a lysis buffer (50mM HEPES (pH 7.4), 150mM NaCl, 10mM EDTA, 4 mM EGTA (pH 7.4), 0.2% NP-40, 5 mM b-glycerophosphate, 100 mM sodium fluoride, 5 mM orthovanadate, 1 mM DTT, 1 mM PMSF and a protease inhibitor) was employed for cell lysis and protein solubilisation. After centrifugation the pellet and supernatant were split and both samples were sonificated, Protein concentration was

determined using a Bradford assay, samples were diluted if necessary and stored as aliquot samples in a sample buffer at -80˚C. The solubilised membrane proteins were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred by electroblotting to a Polyvinlidene Difluoride membrane using Tris-glycine buffer for 2 hr at 25 Volt. Immunoblots were blocked 30 hr with I-block (Tropix) at room temperature. After blocking the blots were incubated withP-glycoprotein monoclonal antibody (MAb) C219 (Alexis, Enzo life sciences 1:1000) overnight at 4˚C, a MAb against P-gp. Horseradish peroxidase-conjugated goat anti mouse gG (1:5000 Santa Cruz) was used as secondary antibody . Detection was carried out with enhanced chemiluminiscence (ECL Pierce) according to the manufacturers instructions. and a digital image was analyzed by quantification of optical density.

For immunohistochemistry (ICH) serial sections of the right hemisphere were cut using a cryostat into 30µm slices and stored in 0.01M PBS (pH 7.4) with 0.01% Sodium Azide. The immunostainings were performed on free-floating sections under continuous mild agitation. Brain sections were washed in 0.01 PBS pH 7.4 and endogenous peroxidase activity was reduced with 0.3% H_2O_2 in 0.01M PBS (pH 7.4) for 30 min at room temperature.The brain sections were washed in 0.01M PBS (pH 7.4) and Preincubated with 5% normal goat serum and 0.1% TritonX-100 in 0.01M PBS (pH 7.4) for 30 min at room temperature, and then incubated with the MAb P-glycoprotein C219 (1:1000, Alexis, Enzo Life Sciences) overnight at 4˚C. Subsequently, sections were rinsed in 0.01 M PBS (pH 7.4) and incubated for 2 h at room temperature with the secondary antibody horseradish peroxidase-conjugated goat anti-mouse (1:400) in 0.01M PBS (pH 7.4) with 0.1% Triton-X 100 and 1% NGS. Then, avidin-biotin complex (1:500 Vector ABC kit, Vector Laboratories, Burlingame, Ca, USA) was added for 1.5 hr at room teperature and washed in 0.01M PBS (pH 7.4), after which the staining was visualized with 1 mg/ml diaminobenzidine and 0.001% H_20_2. Thereafte, sections were rinsed in 0.01M PBS (pH 7.4), mounted on slides (Superfrost) with 1% Gelatine and 100 mg potassium chromium III sulfate, dehydrated by the ethanol-xylol serie and coverslipped for microscopic analysis.

Results

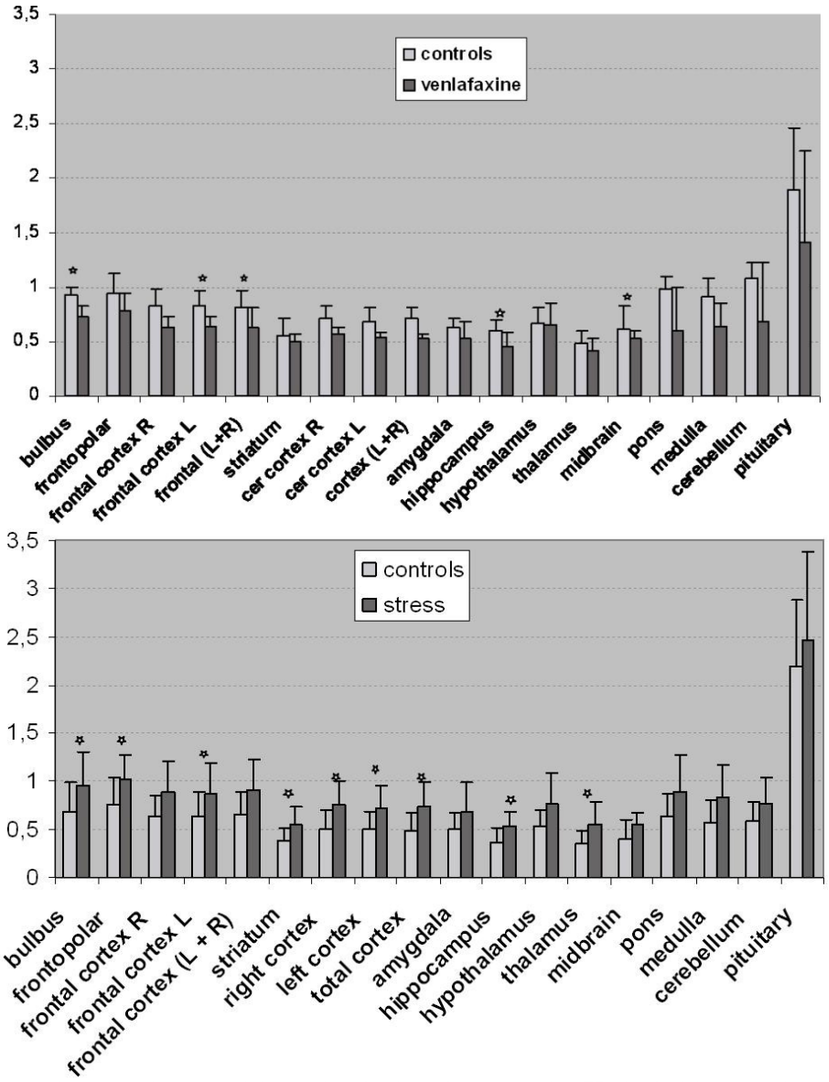
Venlafaxine (PET study)

The image files of 5 venlafaxine treated rats and 5 sham were available for analysis with both a kinetic model (one compartment model) and a graphical approach (logan analysis) (see figure 9.1). There was good overlap between the two methods, pearson's ρ was 0.99 for the controls and 0.88 for the venlafaxine group. ROIs lining extracerebral

structures (i.e. bulbus, frontopolar lobe, pons and medulla) had much higher V_T's than deeper brain structures, which is due to spill-in activity. The mean V_T of controls in all ROIs was higher than in the venlafaxine group, significantly in 6 ROIs and a significant trend was seen in 7 ROIs (see table 9.1). When corrected for multiple testing, V_T was not different for any single ROI (see figure 9.1A).

Figure 9.1

A: Mean distribution volumes (V_T) of venlafaxine vs. controls for each ROI.
B: Mean distribution volumes (V_T) of chronic stress vs. controls for each ROI.



The pituitary lies outside the blood-brain barrier and the V_T is 2.5 larger than the mean V_T. The bars with an asterix (*) differ significantly in V_T. (not corrected for multiple comparisons)

Stress (PET study)

11 controls and 9 rats from the stress procedure were available for the analyses. Logan analysis and one compartment kinetic analysis were carried out. The correlation coefficient ρ for both groups was 1.00. In the kinetic analysis, the V_T in 9 ROIs was significantly higher in the stress group, and in the remaining 8 ROIs a significant trend was seen (see table 9.1, figure 9.1B). The pituitary, with little or no Pgp expression, was the only area showing no difference at all between groups (p = 0.47). Again, areas lining the extracellular structures had a higher V_T, caused by spill-in. In none of the ROIs a significant difference was seen after Bonferroni correction.

Table 9.1

t-tests (logan analysis) for regions of interest (PET)

ROI	venlafaxine	stress
	p	p
bulbus	0,011	0,064
frontopolar	0,086	0,036
frontal cortex R	0,060	0,035
frontal cortex L	0,039	0,079
frontal cortex (L + R)	0,048	0,039
striatum	0,086	0,023
right cortex	0,058	0,019
left cortex	0,15	0,023
total cortex	0,091	0,013
amygdala	0,031	0,079
hippocampus	0,55	0,019
hypothalamus	0,16	0,050
thalamus	0,047	0,028
midbrain	0,25	0,058
pons	0,073	0,096
medulla	0,091	0,053
cerebellum	0,030	0,077
pituitary	0,129	0,47

ROI: region of interest; p = p-values, none is significant after correction for multiple testing

Western immunoblots of hippocampus

We utilized MAb C219 and performed Western immunoblot analysis of extracts of hippocampus. Three different animal models were compared with control rats: 1) neuroinflammation model (Herpes Simplex Virus Encephalitis), 2) chronic stress model and 3) model for chronic administration of an antidepressant. In homogenates from rat hippocampus two immunoreactive bands were detected , a major band at 140 kDa and a very weak one at 174 kDa. Both bands represent isoforms of P-gp, the 140 kDa being the main isoform of P-glycoprotein in the rodent brain,[3] The 174 kDa was too weak to be analyzed with densitometry (figure 9.2A). Therefore comparisons were made on the

basis of the 140 kDa band (figure 9.2B. It is to note that no actin band was included in the blots, which is necessary as reference value for comparison. Nevertheless P-gp expression seems increased with neuroinflammation, and decreased with chronic stress. The venlafaxine treated rats did not show differences in P-gp expression, however the bands were of low quality. It is obvious that our preliminary results must be replicated in a larger sample before drawing any conclusions.

Figure 9.2

Western blot analysis of P-glycoprotein using C219 antibody.

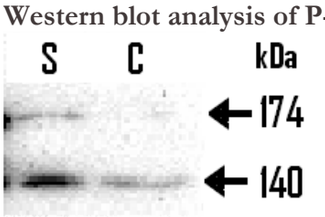


Figure 9.2A showing two bands (stress (S) versus control (C)) that represent the two isoforms of P-gp, found in the BBB. The 174kDa band is the full-length (mature) form. The 'immature' form is the main isoform in rodents, and dominates in situations where P-gp is upregulated. The 140 kDa band represents P-gp (mdr3),

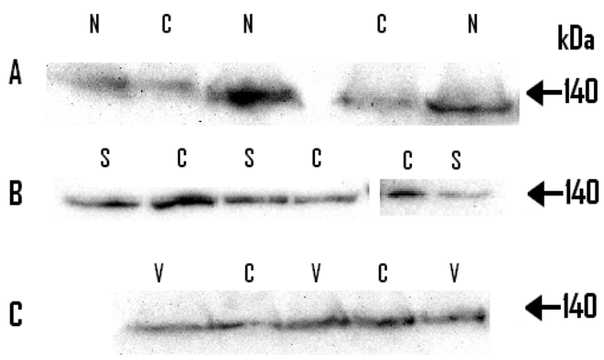


Figure 9.2B showing three conditions compared to controls (C):
A= neuroinflammation versus controls
B = stress versus controls
C= chronic administration of venlafaxine versus controls

Discussion

In a previous PET study with rats it was demonstrated that sustained venlafaxine administration led to an increased P-gp activity at the BBB,[20] whereas chronic stress led

to a decreased activity of P-gp.[20] This study shows that P-gp is globally affected in both situations. Immunochemical studies in rats and PET studies in man suggest that P-gp expression develops synchronously with other BBB components and that P-gp is present in nearly every region (see introduction) of the brain.[7,8,22] The uniform distribution of P-gp in the brain is a logic explanation for the global changes in P-gp activity. In depressive disorders and schizophrenia, both stress related disorders, regional changes in P-gp activity have been reported,[10,11] which seem to be in contrast with the results of regional analysis of the animal PET study. However, only 5 areas in the PET study on depressive disorders were analyzed, compared to 18 areas in the animal study. Besides, the methodology in both studies differed. If the same (small number of) areas had been chosen in the animal study, there would be a trend towards a significant decrease in P-gp activity in the hippocampus and (total) cortex. A regional change in P-gp activity in chronic stress can therefore not be excluded on the basis of these results.

The changes in P-gp activity may reflect changes in protein regulation at various levels. Although preliminary, the first results of the western blots support the PET results. The P-gp expression in neuroinflammation seems increased compared to controls, which is in line with the PET results[21] showing an increased P-gp activity throughout the brain (except in cerebellum and brain stem) in an acute neuroinflammation. In contrast, P-gp expression might be lower at the BBB, after exposure to chronic stress, which is contingent with decreased P-gp activity. However, comparison of different samples is not reliable without an actin band, which serves as reference measure for the amount of protein in a sample. The quality of the venlafaxine blots was too poor to be analyzable.

Thus, the preliminary results of this study suggest that changes in P-gp activity due to chronic antidepressant treatment (venlafaxine) and chronic stress are not confined to specific regions in the brain, which is in line with the uniform distribution of P-gp in the brain. Moreover, the changes in P-gp activity may be connected to regulatory changes in protein level, even after 4 days.

To underpin these preliminary results, the western blot analysis of all samples, including frontal cortex samples must be finished, and these results must be correlated to the individual PET measurements. Immunohistochemistry may support the idea of global downregulation of P-gp in chronic stress in rats, but was not yet performed at the time of writing of this manuscript.

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Chapter 10

General discussion and concluding remarks

Both MDD and schizophrenia are severe psychiatric disorders, both tending to chronicity and affecting many people throughout the world. Schizophrenia is often a debilitating disease, characterized by several relapses with psychotic features, decreased skills during the life cycle, often leading to elimination of major social activities and relations. Neurodevelopmental events and genetic factors may contribute in the pathophysiology.

MDD is considered a heterogeneous condition in which different biologic abnormalities play a role in the symptomatology. The life time prevalence of MDD in the Netherlands is around 15%, the one year prevalence was 5.8% in 1998.[1] Today, MDD is worldwide the third leading cause of disease burden.[2] Despite intensive research during the past decades, the neurobiological basis and the pathophysiology of MDD remain largely unknown, although genetic factors and childhood trauma's are known to play a role.

The aim of the present thesis was to investigate the possible role of P-glycoprotein (P-gp) at the blood-brain barrier (BBB) in major depressive disorder (MDD) and schizophrenia.

Pertaining to the pathophysiology of both disorders, neuroinflammatory events appear to play a central role. For instance, it is now known that stressful events paving the way to stress related disorders, lead to changes in neuroplasticity, impair neurogenesis and may lead to a neuroinflammatory response in the brain.[3] As such, it has been suggested that neurotoxic processes may contribute to the pathophysiology of neurodegenerative diseases. An increase or accumulation of toxic substances in the brain can alter neurotransmission or may damage neurons.[4,5]Accumulation of toxic substances can occur as a result of a diminished blood-brain barrier (BBB) function. Likewise a defective BBB function may cause a hampered outward transport of toxic waste products, leading to malfunctioning neuronal tissue. the A PET study in Parkinson's Disease supported this theory.[6] In this study it was found that the function of P-gp, an important efflux pump in the BBB, was diminished in the midbrain.

The BBB is formed by the brain capillary non-fenestrated polarized endothelial cells that have high-resistance tight junctions. Besides low passive permeability, the brain is protected from potentially harmful endogenous and exogenous substances by efflux transporter proteins, located in the brain capillary wall. P-glycoprotein is a product of the ABCB1 gene and is most principal drug efflux transporter, involved in the outward transport of a wide variety of lipophilic drugs and endogenous substances.[7] Attenuation of P-gp function, for example through use of pharmacologic inhibitors, results in

substantial changes in the pharmacokinetics and pharmacodynamics of various substrates. [8,9]

We hypothesized that P-gp function was altered at the BBB by the effects of neuroinflammation, which appear to play a role in MDD and schizophrenia. In the first study (*chapter 4*), P-gp function at the BBB was investigated in 13 patients with MDD, all using antidepressants, compared to a sex and age matched control group, using [^{11}C]-verapamil as PET tracer. Cerebral distribution volume (V_T) was used as measure for P-gp function. In addition, three (common) polymorphisms were determined with rt-PCR and analyzed as covariate of V_T. Both region-of-interest (ROI) and voxel analysis using statistical parametric mapping (SPM2) were performed to assess regional brain P-gp function. We found a significantly decreased V_T (i.e.[^{11}C]-verapamil uptake) in patients with MDD, with both methods, representing an increased P-gp function, mainly in prefrontal and temporal regions. A genotype effect was not seen in this small sized group. In a post hoc analysis it appeared that 7 out of 13 patients were considered to have a treatment resistant depression (TRD). Comparison between the subgroups (TRD vs. non-TRD), was not eligible since these groups were not matched. The question of biased results (increased activity of P-gp) related to TRD could not be excluded. The study had several limitations. Probably the most serious limitation concerned the missing of a metabolite analysis. Without this input function it can not be excluded that the difference between the groups is not related to P-gp function but to a different rate in metabolism of the tracer. In *chapter 4* we have discussed why we assume metabolism has no major influence on P-gp functionality. Other drawbacks were the use of several antidepressants and other psychotropics, some of them with an inconclusive effect on P-gp. In *chapter 5* the same protocol is used to assess the in vivo function of P-gp in 10 schizophrenic patients, compared to a matched control group. The results were very much comparable to the results in the MDD study, although the results of the voxel-wise analysis were only significant when small volume correction was applied. Nevertheless, the results in both studies suggested a regional increase in P-gp function, which might denote a general disease characteristic (eg. inflammation), or be related to medication. Although we excluded the use of inhibitors of P-gp at recruitment, both antidepressants and antipsychotics have shown (low) affinity (principally in *in vitro* studies) to P-gp. In addition, some appear to exhibit inhibition of P-gp activity *in vitro*.[10-13] The *in vivo* inhibitory effect on P-gp in human is unknown, and presumed to be negligible, because supratherapeutical doses were needed to inhibit P-gp in BBB models.[12] In a mouse model Grauer and Uhr showed that long term administration of a weak to moderate P-gp substrate (amitriptyline) does admittedly not lead to significant differences between Pgp knock-out and wild type mice, but led to large differences in brain accumulation of its metabolites.[14]

The effects on P-gp activity of both medication, MDD, schizophrenia and stress we found (*chapters 4,5, and 8*) could have been influenced by gene variation as well. Although we determined three polymorphism in the human PET studies, the sample was expectedly not adequate to detect small gene effects. The literature on ABCB1 polymorphisms relating gene variation to phenotype is far from conclusive. In the following *chapters 6 and 7* we present the results of the NESDA study, a large multicenter cohort, investigating the long term outcome of depression (and anxiety) as it presents in the general population. Based on our previous results (*chapters 4,5 and 8*), a decline of P-gp functionality in chronic stress, we hypothesized that ABCB1 might be a susceptibility factor in depression. Furthermore, we were interested in the relation between antidepressant related side effects and ABCB1 polymorphisms. Data on dose-to-plasma concentrations were not available. We examined the possible association of 6 SNPs (single nucleotide polymorphism) (i,e, three common SNPs and 3 SNPs having shown a positive association on treatment response[15,16]) with possible susceptibility factors for depression. Data from meta analyses suggested that familial aggregation in MDD was particularly related to 'early onset' (often defined as 'beginning before the age of 30') and recurrent episodes (i.e. more than 1 episode).[17] For two of SNPs that had shown to be related to faster response to an antidepressant (with affinity for P-gp)[15,16] a negative association was found for the age of onset, amounting to a smaller change on MDD starting at young age, for those with the mutation (rs2235040, rs2232583, rs1045642). Furthermore, an association was found between the haplotype 1236T-2677T-3435C and recurrent episodes. The Odds ratios were small (0.735-0.785), and the fact that they were found in a large sample (n=1826), indicate that these polymorphisms will not be not useful as single indicator in clinical practice. Nevertheless, the uncovering of associations in *ABCB1* and other genes, each conferring a small effect on the MDD phenotype may help to point out susceptibility factors in the disorder. Another important finding in the NESDA study was the association of two *ABCB1* polymorphisms rs2235040 and rs2032583 with the incidence and severity of side effects related to SSRI's (that are P-gp substrates). Also, a haplotype, containing the three most common coding SNPs (i.e. 1236C>T, 2677G>T, 3435C>T) and the two SNPs rs2235040 and rs2032583 (1236T-2677T-3435T-rs2235015G-rs2032583T-2235040G) was found to be associated with occurrence of adverse effects. Occurrence of anticholinergic and serotonergic adverse effects was also predicted by these SNPs and haplotype. In particular, these SNPs and haplotype were associated with sleeplessness. Our results suggest that these polymorphisms may predict the occurrence of (anticholinergic and serotonergic) side effects in patients who use a SSRI with affinity for P-gp. The linkage of SNP rs2032583 to the haplotype 1236T-2677T-3435T may act as a marker of a functional haplotype of *ABCB1*. Of course, caution must be exercised with the interpretation of these results, as there are many controversies on ABCB1 related gene effects.

In *chapter 8*, which is a sequel to the studies described in *chapters 4 and 5*, a comparison was made between two of the presumed explanations of increased cerebral P-gp activity. In male Wistar rats, we compared the effect of chronic stress and the effect of chronic administration of venlafaxine to control rats. A 3-week foot shock procedure was used as a model for human depression. [^{11}C]-verapamil-PET was used to assess P-gp function *in vivo*. Venlafaxine exhibits weak to moderate affinity as a P-gp substrate[18,19] and does not modulate cyp3A4, the enzyme involved in [^{11}C]-verapamil metabolism. Although is was not feasible to perform metabolite analysis in the animals in this study, we corrected for metabolism by using data on [^{11}C]-verapamil metabolism from another experiment. To test whether the outcome variable (V_T) was influenced significantly by changes in metabolism, we constructed metabolite curves, either representing slower or faster metabolism and used these as input for the analysis. Only slight changes were seen, which made an effect due to metabolite changes less probable. Both groups differed significantly from their control group: chronic administration of venlafaxine had led an increased activity of P-gp, the whole brain being the region-of-interest, while chronic stress gave the opposite effect: cerebral P-gp activity was significantly decreased. Thus, on the one hand, the findings implicate that chronic stress (as a model for human depression) cause diminished activity of P-gp, which is in line with the cytokine hypothesis of depression. In such a model depression is seen as a neuro-inflammatory event, in which proinflammatory cytokines cause damage to the BBB and elicit P-gp inhibition,[20-22] In case of chronic stress, however, a few (*in vitro*) studies suggest that P-gp is increased.[23,24] To date, there are no *in vivo* studies to confirm any modulatory effect by chronic stress. On the other hand, the results implicate that chronic administration of a P-gp substrate (venlafaxine) causes an increase in P-gp function. The effects of both venlafaxine and chronic stress on P-gp may indicate an up-and downregulatory phenomenon respectively. The studies of Uhr *et al.* and Grauer and Uhr may support the idea of upregulation of P-gp after chronic administration of a P-gp substrate, since they registered (in two different studies) a significantly decreased brain uptake in wild type mice compared to knock-out mice (acute effect), that was no longer present in mice after long term administration of amitriptyline (see figure 10.1).[14,25]

In *chapter 9* the results of *chapter 8* are further elaborated. We hypothesized that the difference in V_T due to chronic administration of [^{11}C]-venlafaxine, would be global in stead of regional, which was the case. P-gp activity was equally distributed throughout the brain, reflecting a homogenous distribution of cerebral P-gp. P-gp was significantly higher in the pituitary, both in the control group and venlafaxine treated group, which fits in the supposition that the pituitary lies outside the BBB.[26] The effect of spill-in in regions adjacent to extracerebral structures caused higher V_T's in both groups. When

these regions (i.e. bulbus, frontopolar lobes, cerebellum, pons, medulla) and pituitary were excluded from the comparison, V_T's, were comparable with the previous study.
We also examined the regional distribution of [^{11}C]-venlafaxine in rats that underwent chronic stress and compared this to the control group. As we had found a regional increase in P-gp activity in patients with a depressive episode, using antidepressants, we hypothesized to find this in the stress experiment too. The decrease in the stress group was globally, and again, for both groups higher V_T's were seen in regions neighbouring non cerebral tissue, and in the pituitary. A comparison between the human study (*chapter 4*) and these results may be halted for several reasons. Firstly, the experiment in rats is only a model for human depression, and secondly, there are many methodological differences between the two experiments. Besides, in the human study, only 5 regions, related to the pathophysiology of depression, were chosen for multiple comparisons, in stead of 18 regions in the rats. If we had chosen these regions for multiple comparison in the rat experiment, a trend towards a significant decrease would be seen in hippocampus ($p = 0.094$) and cortex ($p = 0.064$). On the basis of these results, it can not be excluded that P-gp activity is regionally changed (decreased).
Chapter 9 also includes the description of western blot and immunohistochemistry techniques. The rat brains of the experiments described in *chapter 8* were collected after the PET experiment. The hemispheres were divided, the left hemisphere was snap frozen, the right was fixed in paraformaldehyde and also cryopreserved. Rat brains from a neuroinflammation experiment were provided (by Janine Doorduin) for comparison with the other pathological conditions and controls. Western blot is used to determine the amount of protein (P-glycoprotein) in brain tissue, and the results can be correlated with the PET results, reflecting the activity of P-gp *in vivo*. In this way a differentiation can be made between increased/decreased pump activity due to up- or downregulation (i.e. increased or decreased amount of protein) or altered pump activity without a change in the amount of protein. Immunohistochemistry enables determination of the amount of protein in specific regions. Accordingly, a comparison between regional P-gp activity and regional amount of P-gp can be made. At this stage, the *in vitro* experiments are in full progress, and results are not available yet.

Concluding remarks and future directions

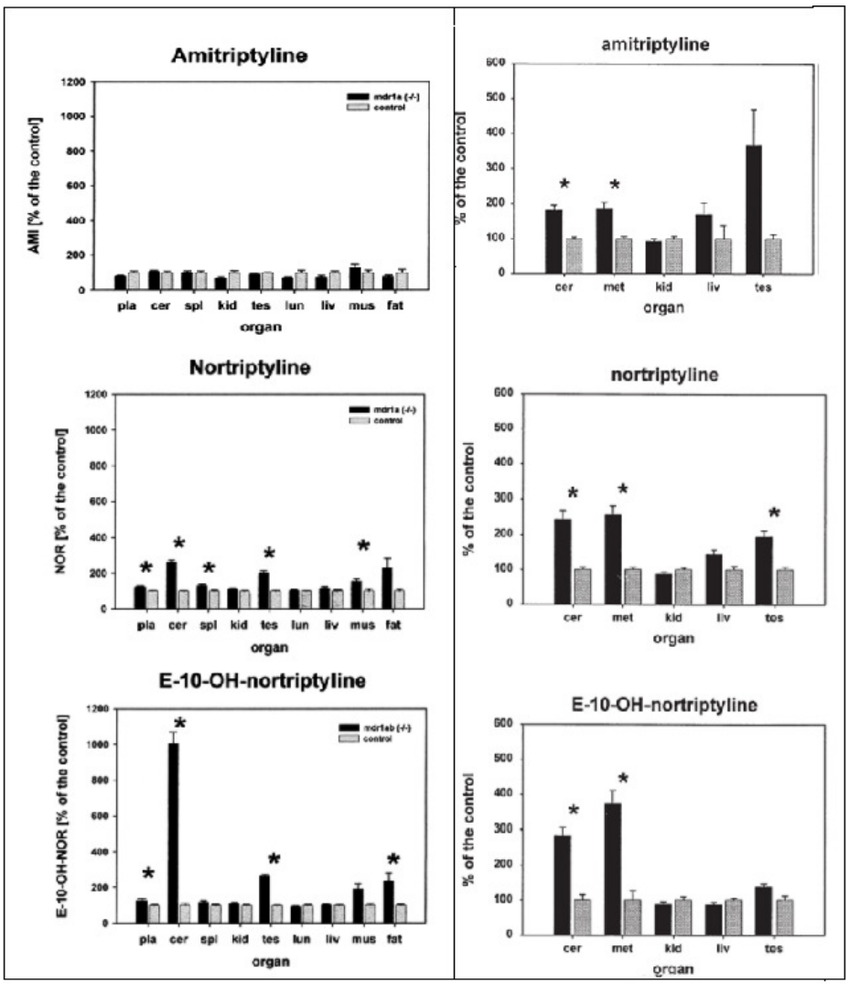
The protection of the brain by the blood-brain barrier has been regarded as a sanctuary for many decades. Intensive research in the past several decades has contributed to a better understanding of the way molecules are transported into the CNS. It has become clear that the transporter P-glycoprotein is involved in the outward transport of many molecules that play a role in depressive disorders, although its role for certain compounds (eg. cortisol) is still debated. Besides, it has been shown that P-gpfunction can be modulated by many factors, which may also play a role in depressive and psychotic disorders. Our results suggest that P-gp activity at the BBB is decreased when

exposed to chronic stress (as model for MDD), and that administration of an antidepressant (a P-gp substrate) increases its function. It is however not clear whether modulation of P-gp is detrimental or has consequences for drug dosing. P-gp function may be decreased as a required step in the acute stress response, necessary to allow the passage of proinflammatory proteins. In the recovery period of a depression the activity of P-gp may then be at another rate. This hypothetical role in different phases during the course of a depressive episode may tested with new PET studies. In this case it would be necessary is to perform PET scans, using different radioligands, aimed at mapping P-gp activity and inflammation. Maybe a more important development in PET research in respect to P-gp imaging is the development of a tracer or a method to study the effect of drug interactions with P-gp. New strategies that could be worked out are, such as radiolabeling of a new drug candidate after administration of an inhibitor, or a double PET scan to evaluate the effect of a P-gp modulator.

Another finding presented in this thesis, is the fact that the gene encoding for P-gp may be related to phenotype. In the first place polymorphisms appeared to be associated with severe side effects on the SSRIs (venlafaxine, citalopram, paroxetine, fluvoxamine) that have shown affinity to P-gp. If these findings are replicated, it shall be a further step towards so called personalized medicine, which involves the avoidance of unnecessary drug trials. Future goals in this regard are the optimalization of a predicted drug response for each individual.

Secondly, three ABCB1 polymorphisms were associated with a lower change on early onset MDD. This finding – if replicated - may contribute in the uncovering of a genetic profile conferring susceptibility for MDD. More large scale genetic studies may help to shed more light on the associations between MDD phenotype and ABCB1 and other genes.

Figure 10.1



Concentration of amitriptyline and its metabolites in various organs in abcb1ab-/- mice (black bars) versus wild type mice (grey bars). At the right, Amitriptyline is once administered 3 hours prior to decapitation, resulting in significantly higher intracerebral concentrations for amitriptyline and its metabolites[25]
At the left, amitriptyline has been administered for 10 consecutive days, resulting in equal intracerebral concentrations for the parent drug, whereas the intracerebral concentration of metabolite E-10-OH-nortriptyline has increased with roughly 250%.[14,25]

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Nederlandse samenvatting algemene discussie en conclusie

Het doel van dit proefschrift is het onderzoek van de mogelijke rol van de eiwitpomp P-glycoproteïne (P-gp), dat aanwezig is in de bloed-hersenbarrière (BHB) in depressie en schizofrenie.

Zowel depressie (MDD, Major Depressive Disorder) als schizofrenie zijn ernstige psychiatrische stoornissen, die wereldwijd voorkomen en gekenmerkt worden door een chronisch beloop.
Schizofrenie is vaak een invaliderende ziekte, die gekenmerkt wordt door terugkerende psychotische episoden en afname van cognitieve en sociale vaardigheden. Vaak treedt er verlies van zowel sociale als maatschappelijke rollen. Afwijkingen in de normale ontwikkeling van het zenuwstelsel en ook genetische factoren kunnen een rol spelen in het ontstaan van deze ziekte.

Depressie wordt beschouwd als een heterogene stoornis, waarbij verscheidene biologische afwijkingen een rol kunnen spelen in het symptomencomplex. De 'life time' prevalentie van depressie in Nederland is ongeveer 15%, terwijl de eenjaarsprevalentie in 1998 5.8% was.[1] Volgens een WHO rapport staat depressie qua omvang en leed tegenwoordig wereldwijd op de derde plaats.[2] Ondanks intensief wetenschappelijk onderzoek sinds de laatste decennia, zijn kennis over het ontstaan en oorzaken van de ziekte grotendeels nog onopgehelderd. Wel is bekend dat genetische factoren en psychotrauma's in de kinderleeftijd een rol kunnen spelen.

Wat betreft de onderliggende ziekteverschijnselen, geldt voor beide stoornissen dat sprake is van ontstekingsreacties in het centrale zenuwstelsel. Stressvolle gebeurtenissen kunnen leiden tot aan stress gerelateerde stoornissen (zoals depressie). Ook bij schizofrenie speelt stress een belangrijke rol. Ten gevolge van stressreacties kunnen allerlei veranderingen optreden in het brein, zoals veranderingen in de plasticiteit van zenuwcellen, afname van nieuwvorming van zenuwweefsel en ontstekingsverschijnselen in het brein.[3] Een mogelijke verklaring voor het ontstaan van beide ziekten wordt gevormd door de hypothese dat ontstekingsreacties in het brein ontstaan door schadelijke stoffen die (onder invloed van stress) het brein binnendringen. Deze kunnen vervolgens bijdragen aan het ontstaan van neurodegeneratie (stoornissen waarbij sprake is van verlies of achteruitgang van mentale functies, waartoe zowel depressie als schizofrenie soms worden gerekend). Ophoping van schadelijke stoffen in het brein kan optreden als gevolg van een verminderde functie van de BHB.[4,5] Een PET-studie (positron emission tomography, dit is functioneel beeldvormend onderzoek) bij mensen met de ziekte van Parkinson ondersteunt deze theorie.[6] In deze studie vond men een afgenomen pompfunctie van P-gp, een belangrijke pomp in de BHB, die vanuit het hersenweefsel moleculen

terugpompt in de bloedbaan. Deze verminderde pompfunctie was gelokaliseerd in hetzelfde hersengebied gelokaliseerd als de ziekte van Parkinson.

De BHB is aanwezig in de kleinste bloedvaatjes van de hersenen (capillairen). Deze capillairen zijn zo gebouwd dat vrijwel geen enkel molecuul doorgelaten wordt. De vaatwandcellen hebben nauwelijks openingen en zitten tegen elkaar aangekit door speciale strengen "tight junctions"). Kleinere moleculen kunnen door diffusie het brein binnenkomen. Mogelijk schadelijke stoffen, zowel lichaamseigen als lichaamsvreemde, worden door naar buiten gerichte pompen (effluxpompen) teruggepompt in de bloedbaan. P-glycoproteïne is de belangrijkste effluxpomp in de BHB. P-gp wordt door het ABCB1-gen gecodeerd. Deze pomp heeft een enorme capaciteit, maar het meest opmerkelijke aan deze pomp is dat het talloze geheel verschillende moleculen kan transporteren.[7] Een afgenomen (P-gp)pompfunctie, bijvoorbeeld door een farmacologische remmer, kan leiden tot een sterk toegenomen hersenopname van moleculen die door P-gp getransporteerd worden ('substraten').[8,9]

Wij veronderstelden dat de functie van P-gp veranderd zou zijn bij mensen met een depressie en schizofrenie, mogelijk ten gevolge van de ontstekingsreactie in het brein.
In de eerste studie (hoofdstuk 4) werd de functie van P-gp onderzocht in 13 mensen met een depressie. Allen gebruikten een antidepressivum. De groep werd vergeleken met een qua leeftijd en sexe gematchte controlegroep. [^{11}C]-verapamil werd gebruikt als tracer. Het distributievolume (V_T) werd gebruikt als maat voor de activiteit van P-gp. Er werd bloed afgenomen voor genetisch onderzoek. Eventuele genvariatie van drie veel voorkomende polymorfismen (van ABCB1) werden als covariaat van het distributievolume geanalyseerd. Zowel een statische (region-of-interest) als dynamische analyse (voxelwise analysis) werd gedaan, deze laatste met behulp van het programma SPM2 ("statistical parametric mapping"), om de regionale P-gp functie te bepalen. We vonden een significante verlaging van het distributievolume, hetgeen correspondeert met een toegenomen functie van P-gp. Deze bevinding werd gedaan met beide methoden, en was met name gelokaliseerd in de prefrontale schors en temporale kwabben, gebieden die beiden geassocieerd zijn met depressie. We vonden geen relatie met variatie in genotype, hetgeen ook niet te verwachten was in zo'n kleine groep. In een posthoc analyse bleek dat 7 patiënten een therapie resistente depressie (TRD) hadden. Een vergelijking tussen de zo ontstane subgroepen (TRD versus non-TRD) liet geen verschillen zien, maar was formeel ook niet mogelijk omdat de groepen niet gematcht waren. Daarmee kon de mogelijkheid van bias van de resultaten door therapieresistentie niet weerlegd worden. Het was dus mogelijk dat de toegenomen P-gp activiteit gerelateerd was aan therapieresistentie, hetgeen zeer belangwekkend zou zijn, aangezien hiermee een verklaring voor therapieresistente depressie gegeven kon worden: doordat P-gp activiteit toegenomen was, kon het

antidepressivum onvoldoende werkzaam kunnen zijn in de hersenen, omdat het in versterkte mate uitgepompt werd door P-gp. De studie kende verschillende tekortkomingen. In de eerste plaats was geen metabolietanalyse verricht, hetgeen de interpretatie van de resultaten bemoeilijkt. Zonder deze inputfunctie kan niet worden uitgesloten dat de verschillen tussen de groepen veroorzaakt worden door verschillen in metabolisme van de tracer. In *hoofdstuk 4* beargumenteren we waarom we aannemen dat metabolisme geen belangrijke invloed heeft gehad op de activiteit van P-gp. Een andere belangrijke beperking van de studie betrof het gebruik van verschillende antidepressiva en andere farmaca bij de onderzoeksgroep. Van sommige farmaca kon niet worden uitgesloten worden dat ze de P-gp activiteit beïnvloedden. In *hoofdstuk 5* werd hetzelfde protocol gebruikt om de *in vivo* functie van P-gp in schizofrenie te bepalen. Dit is gedaan in 10 patienten en 10 gematchte controlepersonen. De resultaten kwamen in sterke mate overeen met de resultaten van de depressiestudie, al was het resultaat van de dynamische analyse alleen significant wanneer de analyse alleen de gebieden van potentieel belang betrof ("small volume corrrection"). De resultaten uit beide studies suggereerden dat sprake was van een regionale toename van P-gp activiteit, hetgeen theoretisch zou kunnen passen bij de ziekte, m.a.w. de lokaal verhoogde activiteit van de pomp zou een ziektekenmerk (bijv. een ontstekingsreactie) kunnen zijn. Ook zou de verhoogde activiteit te verklaren kunnen zijn door het gebruik van medicatie. We hadden weliswaar het gebruik van medicatie met een duidelijk remmende werking op P-gp uitgesloten voor deelnemers aan de studie, zowel antidepressiva als antipsychotica blijken echter in celstudies lage tot matige activiteit te hebben voor P-gp. Bovendien blijken sommige middelen ook nog een remmend effect op P-gp te kunnen hebben in zulke celstudies.[10-13] Het is niet bekend of dit effect ook in de mens zal kunnen optreden, en waarschijnlijk is het effect te verwaarlozen, omdat het remmende effect in deze cellijnen (modellen voor de BHB) pas optrad bij concentraties die ver boven het therapeutische niveau lagen.[12] Toch is het niet geheel uit te sluiten dat medicatie de verschillen in P-gp activiteit veroorzaakte. In een diermodel (muizen) toonden Grauer en Uhr aan dat langdurige toediening van amitriptyline (een antidepressivum met een zwak tot matige affiniteit voor P-gp) geen verschil in concentratie gaf in de hersenen bij muizen zonder P-gp in de hersenen ("knock out" model) ten opzichte van genetisch normale muizen ("wild type"), terwijl de metabolieten van amitriptyline juist in sterke mate waren opgehoopt in de hersenen bij het knock out model.[14]

De gevonden effecten op de activiteit van P-gp door zowel medicatie als depressie, stress en psychose (zie *hoofdstuk 4,5, en 8*) kunnen ook beïnvloed zijn door genetische variatie. Alhoewel we in de humane PET studies geen aanwijzingen vonden voor genveranderingen in drie polymorfismen (3435T>C, 2677G>T/A, -129T>C), is daarmee nog geen uitspraak te doen over een relatie tussen de gevonden P-gp veranderingen in

functie en genvariatie, omdat de getoetste sample veel te klein was. De literatuur betreffende de relatie tussen het *ABCB1* gen en fenotype is alles behalve eensluidend. In de *hoofdstukken 6 en 7* presenteren we de resultaten van de NESDA studie (NEtherlands Study on Depression and Anxiety). DE NESDA studie is een groot multicenter cohort onderzoek, dat de lange termijn uitkomsten van depressie (en angststoornissen) onderzoekt, zoals deze stoornissen zich in de algemene populatie voordoen. Op basis van de resultaten, gepresenteerd in *hoofstuk 4,5 en 8,* namelijk dat sprake is van een verlaagde activiteit van P-gp in depressie, was onze hypothese dat genetische variatie van het ABCB1 gen geassocieerd zou kunnen zijn met een verhoogde kwetsbaarhied voor het ontstaan van depressie, ofwel het beloop van depressie. Verder waren we geïnteresseerd in de mogelijke relatie tussen *ABCB1* polymorfismen en bijwerkingen op antidepressiva (P-gp substraten). We onderzochten de mogelijke associatie van 6 SNPs (single nucleotide polymorphisms) met mogelijke kwetsbaarheidsfactoren voor depressie. Drie veel voorkomende SNPs en drie polymorfismen, voor welk een relatie was aangetoond met respons op antidepressieve behandeling bij depressie[25,26] werden geanalyseerd. Data uit meta-analyses hebben laten zien dat familiair voorkomen van depressie met name voorspeld kan worden door het voorkomen van recidiverende depressieve episoden (het voorkomen van meer dan een episode) en een vroeg ontstaan van de ziekte (beginleeftijd voor het 30^{e} jaar).[27] Voor 2 van de onderzochte SNPs (rs2235040 en rs 2032583) vonden we een negatieve associatie met het vroeg ontstaan van depressie, hetgeen impliceert dat een persoon met het recessieve allel van een van deze SNPs beschermd is tegen het vroeg optreden van depressie. Voor 3435CC vonden we een zelfde verband, dat niet significant was na correctie voor het aantal tests. Er werd ook een significant verband gevonden tussen het haplotype 1236T-2677T-3435C en het aantal recidief depressies. De Odds ratio's waren klein (0.735 – 0.785), hetgeen aangeeft dat deze polymorfismen niet bruikbaar zijn als enkele indicator voor het bepalen van kwetsbaarheid in een individu. De gevonden verbanden hebben elk afzonderlijk een kleine invloed op het fenotype. Niettemin is het vinden van dergelijke associaties van groot belang voor het verder in kaart brengen van kwetsbaarheidsfactoren van de depressieve stoornis.

Een andere belangrijke bevinding in de studie met de NESDA, beschreven in *hoofdstuk 7*, was de associatie tussen twee *ABCB1* polymorfismen (rs2235040 en rs2032583) en het optreden (incidentie) en de ernst van bijwerkingen gelateerd aan SSRIs (selectieve serotonine heropnameremmers), die affiniteit hebben voor P-gp. De twee SNPs voorspelden ook significant de ernst van anticholinerge en serotonerge bijwerkingen. De relatieve overrepresentatie van paroxetine (>35%) in de sample kan verklaren waarom een relatie gevonden werd met anticholinerge bijwerkingen. Een verklaringsmechanisme kon ook voorspeld worden in paroxetine (n=110), een SSRI met meer anticholinerge bijwerkingen dan andere SSRI's, hetgeen mede kan verklaren dat juist op dit middel een verband gevonden wordt. Voor venlafaxine, dat minder

anticholinerge werking heeft dan paroxetine, vonden we een significante trend naar een verband tussen anticholinerge bijwerkingen en rs2235040 en rs2032583. Voor citalopram, dat vrijwel geen anticholinerge bijwerkingen heeft, werd geen effect gevonden. Onze resultaten suggereren een verband tussen het optreden en ernst van (anticholinerge) bijwerkingen op SSRI's (indien P-gp substraat) en ABCB1 genpolymorfismen. De vele tegenstrijdige resultaten tot op heden manen tot voorzichtigheid in de interepretatie van onze bevindingen.

In hoofdstuk 8 wordt een dierstudie beschreven, waarin we zoeken naar de verklaring van de resultaten beschreven in *hoofdstuk 4 en 5*. De regionale verhoging van P-gp activiteit zou verklaard kunnen worden door de stoornis (i.e. stress als pathofysiologisch mechanisme) of door het gebruik van medicatie. In verschillende groepen ratten vergeleken we (ten opzichte van een controlegroep) het effect van chronische stress en het effect van langdurig gebruik van het antidepressivum venlafaxine. Met een procedure, waarbij ratten gedurende 3 weken werden blootgesteld aan elektrische schokken, werd een depressie nagebootst. [^{11}C]-verapamil-PET werd gebruikt om de activiteit van P-gp *in vivo* te bepalen. Venlafaxine heeft zwakke tot matige affiniteit voor P-gp[15,16] en heeft geen modulerende invloed op cyp3A4, het enzym dat grotendeels [^{11}C]-verapamil metaboliseert. Alhoewel het niet mogelijk was in de proefdieren een metabolietanalyse te verrichten, werd in deze studie gecorrigeerd voor het metabolisme door [^{11}C]-verapamil metabolisme data te gebruiken van een andere studie. We hebben hierbij gekeken naar eventuele gevolgen van veranderingen in het metabolisme door de metabolietcurve te manipuleren. Het bleek dat een iets langzamer of iets sneller metabolisme geen grote veranderingen op de uitkomstmaat (V_T) had, hetgeen het onwaarschijnlijk maakte dat de verschillen tussen de groepen ratten zouden berusten op verschillen in metabolisme. In beide experimenten waren significante verschillen tussen de groepen te zien. In de groep ratten die venlafaxine ontvingen, werd een significante toename van P-gp gezien, terwijl chronische stress juist leidde tot een significante verlaging van P-gp. De verschillen werden gezien op 'whole brain' niveau. De betekenis van deze bevindingen is dat chronische stress (als model voor depressie) leidt tot een verminderde pompfunctie van P-gp, hetgeen ondersteund wordt door de 'cytokine hypothese' in depressie. Hierin wordt depressie gezien als resultante van een ontstekingsreactie in het centrale zenuwstelsel. Ontstekingsbevorderende eiwitten veroorzaken hierbij schade aan de BHB en zorgen voor remming van P-gp.[17-19] In enkele *in vitro* studies zijn aanwijzingen gevonden voor een toename van de P-gp activiteit door chronische stress.[20,21] Er zijn echter geen in vivo studies die deze hypothese kunnen bevestigen. Onze studie is de eerste in dit opzicht, waarin een verband gezien wordt tussen chronische stress en verandering van de P-gp activiteit. Aan de veranderde activiteit in zowel chronische stress en na chronische toediening van venlafaxine kan

respectievelijk een af- of toename van de hoeveelheid P-glycoproteïne ten grondslag liggen, zgn. down- of upregulatie. De studies van Uhr *et al.* en Grauer en Uhr ondersteunen deze gedachte. In twee studies toonden zij aan dat een enkele gift amitriptyline (een P-gp substraat) in muizen leidde tot een significant verlaagde hersenopname in genetisch normale muizen (wild type) ten opzichte van muizen die P-gp misten ('ABCB1a -/- knock out' muizen). Wanneer echter gedurende 10 dagen amitriptyline toegediend werd, werd geen verandering meer gezien tussen beide groepen in de concentratie amitriptyline in de hersenen (zie figuur 10.1).[14,22]

In *hoofdstuk 9* worden de resultaten van *hoofdstuk 8* verder uitgewerkt. We veronderstelden dat chronische toediening van [^{11}C]-venlafaxine niet zou leiden tot regionale verschillen in distributievolume (V_T) (als maat voor P-gp activiteit, het V_T is omgekeerd evenredig met de P-gp activiteit), maar dat de verschillen alleen globaal zouden zijn, hetgeen inderdaad het geval was: in alle regio's werd een vergelijkbare afname van V_T gevonden. Het V_T was in de hypofyse veel hoger in beide groepen, hetgeen een bevestiging is van de veronderstelling dat de hypofyse 'buiten' de bloed-hersen barrière ligt.[23] Daarnaast was sprake van toegenomen V_T waarden in de gebieden grenzend aan niet-hersenweefsel, dat niet door verlaagde P-gp activiteit verklaard werd, maar als gevolg van 'overstraling' uit deze gebieden gezien werd. Wanneer deze perifere gebieden (i.e. bulbus, frontopolaire kwabben, cerebellum, pons, medulla) niet in de analyse werden meegenomen, waren de distributievolumes vergelijkbaar met de resultaten zoals in *hoofdstuk 8* beschreven.
We onderzochten ook de regionale verdeling van [^{11}C]-venlafaxine in ratten, die blootsgesteld waren aan chronische stress en vergeleken dit met de controlegroep. Een regionaal verschil in distributievolume zoals in de depressiestudie werd niet gevonden, er was sprake van een algemene verhoging van V_T (passend bij een verlaging van P-gp functie) en net als bij de venlafaxinestudie werd er verhoogde activiteit gemeten in de perifere gebieden en in de hypofyse, die geen P-gp in haar capillairen heeft. Een vergelijking met de humane (depressie)studie gaat echter op verschillende fronten mank: ten eerste, is het stressexperiment bij ratten een model voor humane depressie, en zodoende nooit geheel vergelijkbaar. In de humane studie werden bovendien slechts 5 gebieden (gerelateerd aan psychopathologie) geselecteerd voor vergelijking, in plaats van de 18 gebieden bij de rat. Als we deze gebieden ook in het rattenexperiment gekozen hadden voor multipele vergelijking, zou sprake zijn van een trend naar significante verhoging van V_T in hippocampus ($p = 0.094$) en cortex ($p = 0.064$). Op basis van deze studie kan daarom niet uitgesloten worden dat sprake is van een regionale veranderingen (verlaging) in P-gp activiteit t.g.v. stress.

Hoofdstuk 9 bevat ook een beschrijving van experimenten met het hersenweefsel van ratten, die na de studie (hoofdstuk 8) getermineerd werden. Het betreft de 'Western

Blot', een techniek, waarbij de hoeveelheid eiwit (in dit geval P-glycoproteïne) bepaald kan worden. Met deze techniek is het mogelijk te onderzoeken of verschil in pompactiviteit berust op een verschil in het aantal pompjes (up- of downregulatie). Een andere nog toe te passen techniek is 'immunohistochemie', die ook regionale verschillen in eiwithoeveelheid kan aantonen. In dit stadium zijn de analyses niet afgerond, en is definitieve bespreking van de resultaten nog voorbarig. Wel kan gezegd worden dat de voorlopige resultaten de resultaten van de PET analyses ondersteunen: de veranderde functie van P-gp gaat samen met een veranderde expressie van het eiwit in het brein.

Concluderende opmerkingen en richting voor verder onderzoek

De bloed-hersen-barrière (BHB) is lange tijd beschouwd als een rigide structuur om het brein, die passage van vele stoffen onmogelijk maakte. Intensief onderzoek in de laatste decennia heeft bijgedragen aan een beter inzicht in de manier waarop het transport van moleculen via de BHB verloopt.

Duidelijk is dat P-glycoproteïne als transporteiwit betrokken is bij het terugwaartse transport (van brein naar bloedbaan) van vele moleculen, die ook bij depressie een rol spelen. In sommige gevallen is niet geheel opgehelderd welke rol P-glycoproteïne speelt bij het transport van een bepaald substraat speelt, zoals bij cortisol bij voorbeeld. Verder is aangetoond dat de P-gp functie gemoduleerd kan worden door vele factoren, zoals ook factoren die een rol spelen bij psychotische en depressieve stoornissen. Onze resultaten wijzen op een verlaging van P-gp activiteit in de BHB bij blootstelling aan chronische stress in ratten, (als model voor depressie) en toediening van een antidepressivum (een P-gp substraat) doet de functie van P-gp toenemen. Het is niet bekend of zulk een verandering pompfunctie op een of andere manier nadelig is, of dat het consequenties heeft voor de dosis van een antidepressivum. Het is mogelijk dat verlaging van de activiteit van P-gp in de acute fase van de stressreactie, zoals bij de depressieve stoornis, nodig is om passage van pro-inflammatoire eiwitten mogelijk te maken. In de chronische fase of in de herstelfase van een depressieve episode kan de activiteit van P-gp anders zijn. Deze hypothetische functie van P-gp in verschillende fasen van een ziekteproces kan onderzocht worden in nieuwe PET studies, door gebruik te maken van verschillende liganden, die zowel inflammatie als P-gp activiteit in kaart kunnen brengen. Nieuwe strategieën kunnen worden uitgewerkt, zoals het labelen van nieuwe potentiële medicijnen, na toediening van een P-gp remmer, of dubbele PET scans, voor en na toediening van een modulator.

Een andere belangrijke bevinding die in dit proefschrift wordt gepresenteerd, is de relatie tussen het depressiefenotype en het gen dat codeert voor P-gp. Ten eerste bleken ABCB1 polymorfismen geassocieerd te zijn met het optreden en de ernst van bijwerkingen op SSRIs (venlafaxine, citalopram, paroxetine, fluvoxamine), die getransporteerd worden door P-gp. Als deze bevindingen gerepliceerd worden, is het

een stapje vooruit in de richting van de zogenaamde gepersonaliseerde geneeskunde, hetgeen impliceert dat onnodige medicatie trials worden vermeden, en een medicatiekeuze zoveel mogelijk gebaseerd is op het genetische profiel van de patient. Toekomstige doelen in dit opzicht zijn optmalisatie van het voorspelde effect van medicatie voor iedere gebruiker. Ook dienen mogelijke epigenetische veranderingen in het P-gp molecuul bestudeerd te worden.

Ten tweede, drie ABCB1 polymorfismen waren geassocieerd met een lagere kans op een vroeg begin van de depressieve stoornis. Een haplotype was geassocieerd met recidief depressie. Deze bevindingen - mits gerepliceerd - kunnen bijdragen aan blootleggen van het genetisch profiel dat kwetsbaarheid voor depressie beschrijft. Om hier meer inzicht in te krijgen, zijn meer grootschalige studies nodig, die licht kunnen werpen op de relatie tussen het depressiefenotype en ABCB1 en andere genen.

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geweest zonder de samenwerking met de faculteit biologie. De samenwerking tussen Peter Meerlo en Aren van Waarde is hierin cruciaal geweest. Anniek Visser en Tim de Jager hebben het uitvoerende werk gedaan in Haren en bij NGMB. Ook de ondersteuning van Janine Doorduin en Jurgen Sijbesma op NGMB en van Girste Dagyte en Folkert Postema in Haren is van groot belang geweest. De inzet en behulpzaamheid van secretaresses en de MNW-ers, die niet altijd de meest makkelijke patiënten kregen, heb ik steeds gewaardeerd. Een conclusie van dit proefschrift is ook dat mensen met een depressie moeilijker te scannen zijn dan mensen schizofrenie. Bij de *in vitro* studie heeft Wanda Douwenga de Western Blots voor en met me gedaan. Anniek, nu in de rol van AIO, hielp me opnieuw met de analyses van Inveon.

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List of abbreviations

ABC: ATP binding cassette
ADR: adverse drug reaction
ANCOVA: analysis of covariance
ASEC: antidepressant side effect checklist
ATC: Anatomical Therapeutical Classification
ATP: Adenosine TriPhosphate
BBB: blood-brain barrier
BCRP: breast cancer resistance protein
CAPP: Clinical Applications Programming Package
CIDI: Composite International Diagnostic Interview
CNS: central nervous system
CSF: cerebrospinal fluid
Cyp: Cytochrome P450
DNA: deoxyribonucleic acid
DSM-IV: Diagnostic Statistical Manual, 4th edition
EC: endothelial cell
EC_{50}: Median Effective Concentration (required to induce a 50% effect)
ET-1: endotheline-1
GWAS: genome wide association study
HAMD: Hamilton depression (rating scale)
HPA axis: hypotuitary-pituary-adrenal (axis)
HSVE: Herpes Simplex Virus Encephalitis
IL: interleukine
kD: kilodalton
KO: knock out
LAT1: large neutral amino-acid transporter
LD: linkage desequilibrium
L-DOPA: L-3,4-dihydroxyphenylalanine
MAO: mono amine oxidase
MBq: megabecquerel
MDR1: multi drug resistance-1
MINI: Mini Neuropsychiatric Interview
MRI: magnetic resonance imaging
MRP: Multi Resistance Protein
NCBI: National Center for Biotechnology Information
NMDA: N-methyl-aspartic acid
OAT: organic anion transporting
OATP: organic anion transporting polypeptide family
OSEM: ordered subsets expectation maximum
PET: positron emission tomography
P-gp: P-glycoprotein
PTSD: posttraumatic Stress disorder
RNA: Ribonucleic acid
ROI: region of interest

SD: standard deviation
sICAM-1: serum Inter-Cellular Adhesion Molecule-1
SNP: single nucleotide polymorphism
SPM2: statistical parametric mapping
SPSS: Statistical Package for the Social Sciences
SSRI: Selective Serotonin Reuptake Inhibitor
TEER: trans endothelial electric resistance
TNFα: tumor necrosis factor α
UKU: Udvalg for Kliniske Undersøgelser (side effects rating scale)
VLA-4: Very Late Antigen-4
VPM-PET: [^{11}C]-verapamil - positron emission tomography
V_T: volume of distribution
WB: Western Blot
WT: wild type